DETERMINANTS OF EMBRYONIC RESISTANCE TO HEAT SHOCK DURING THE PREIMPLANTATION PERIOD IN CATTLE

By

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Abstract of Dissertation Presented to the Graduate School
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Preimplantation embryos acquire resistance to elevated temperature as they advance in development. The goal of this dissertation was to identify determinants of embryonic resistance to elevated temperature in preimplantation bovine embryos.

Studies were performed to determine the role of heat-induced apoptosis in bovine embryos. Exposure of bovine embryos $\geq$16 cells to heat shock increased the percentage of TUNEL-positive cells and group II caspase activity. However, heat shock did not induce apoptosis in 2- or 4-cell embryos or increase group II caspase activity in 2-cell embryos. Treating embryos with an inhibitor of group II caspases (z-DEVD-fmk) blocked heat-induced increase in caspase activity and TUNEL-positive cells. Exposure of embryos to heat shock reduced development to the blastocyst stage and this deleterious effect of heat shock was magnified in the presence of z-DEVD-fmk, suggesting that blocking apoptosis has a detrimental effect on embryonic resistance to heat shock.

The detrimental effects of heat stress on fertility in cattle are less pronounced in heat-tolerant breeds than heat-sensitive breeds. Exposure of bovine embryos to heat shock decreased development to the blastocyst stage and embryo cell number. However, the deleterious effects of heat shock was less pronounced for Brahman than for Holstein and Angus. In contrast to the sensitivity of embryos to heat shock, there was no effect of heat shock on [^{3}H]leucine incorporation into proteins secreted by oviductal or endometrial explants. Brahman and Senepol lymphocytes were more resistant to heat-induced apoptosis than the other breeds.

In the last study we demonstrated that multiple intramuscular injections of vitamin E and selenium did not improve postpartum reproductive function or milk yield in lactating dairy cows. In vitro studies showed that vitamin E did not alleviate the deleterious effects of heat shock on development to the blastocyst stage.

Taken together, results suggest that the ability to undergo heat-induced apoptosis to remove damaged blastomeres and embryo genotype are important determinants of embryonic resistance to heat shock. In contrast, supplemental antioxidants treatment was not able to improve pregnancy rates in lactating dairy cows or development of heat-shocked embryos.

CHAPTER 1
INTRODUCTION

Mammals are homeothermic. Exposure to elevated ambient temperature activates thermoregulatory regions of the central nervous system to reduce internal heat production and increase net heat loss to the environment. Both the physiological adaptations to maintain a constant body temperature under heat stress conditions, for example reduced blood flow to the uterus (Roman-Ponce et al., 1978), and the increase in body temperature itself, for example, reduced embryo survival (Rivera and Hansen, 2001), can be deleterious to the reproductive process. In cattle, which strive to maintain a core body temperature of 38.5°C, heat stress reduces the duration (Abilay et al., 1975; Wolfenson et al., 1988) and intensity of estrus (Gangwar et al., 1965) and causes a large decline in pregnancy rates per insemination in lactating dairy cows (Badinga et al., 1985; Cavestany et al., 1985; Al-Katanani et al., 1999). In one study, for example, pregnancy rates in lactating cows in Florida were reduced from 40-50% during the cool months of the year to less than 10-20% during the hot months of the year (Badinga et al., 1985).

The increased heat production associated with lactation makes the lactating dairy cow especially susceptible to heat stress. The increase in core body temperature accompanying exposure to heat stress is greater for lactating cows than for nonlactating cows (Cole and Hansen, 1993). Not surprisingly, the summer depression in pregnancy rate in Florida seen in lactating dairy cow was not seen in nonlactating heifers (Badinga et al., 1985). In addition, among lactating cows, those with a higher milk yield have greater reduction in fertility in summer (Al-Katanani et al., 1999). Thus, continued increases in milk yield in dairy cows have the potential for exacerbating effects of heat stress on reproduction. Moreover, adoption by producers of pharmaceutical products that increase milk yield, like bovine somatotropin, will also make cows more susceptible to heat stress. Indeed, heat stressed cows injected with bovine somatotropin had increased rectal temperatures as compared with control cows (Cole and Hansen, 1993). In contrast to the dairy cow, cattle of *Bos indicus* origin, which evolved under tropical and subtropical conditions, experience less severe effects of heat stress on hyperthermia (Adeyemo et al., 1979; Bennett et al., 1985; Hammond et al., 1996; Hammond et al., 1998; Gaughan et al., 1999) and reproduction (Turner, 1982) than *Bos taurus* from temperate regimen. Perhaps genes from these thermotolerant breeds may be used to increase pregnancy rates in dairy cows.

The adverse effects of elevated temperature on fertility is multifactorial since it impairs physiological and cellular functions in different tissues. Heat stress compromises follicular dynamics to reduce the growth of the dominant follicle (Badinga et al., 1993) and the ability of the dominant follicle to exert dominance (Wolfenson et al., 1995; Wilson et al., 1998). This loss of follicle dominance may be related to reduced plasma inhibin concentrations (Roth et al., 2000), increased plasma FSH concentrations (Roth et al., 2000) and reduced plasma concentrations of estradiol 17-β (Wolfenson et al., 1995). Heat stress causes earlier emergency of the second wave dominant follicle (Wolfenson et al., 1995) and such a phenomenon may lead to ovulation of an aged oocyte. Indeed, there is evidence that oocytes are compromised by heat stress.

Oocytes harvested from follicles of Holstein cows during summer had reduced ability to develop to the blastocyst stage after in vitro fertilization than oocytes harvested during winter (Rocha et al., 1998; Al-Katanani et al., 2002b). Moreover, exposure of Holstein heifers to heat stress between the onset of estrus and insemination increased the proportion of abnormal and retarded embryos as compared to heifers maintained at thermoneutratlity (Putney et al., 1989b). Direct effects of elevated temperature on oocyte competence also has been demonstrated. Heat shock decreased the ability of the oocyte to develop to the blastocyst stage (Edwards and Hansen, 1997) and reduced oocyte protein synthesis (Edwards and Hansen, 1996). Therefore, both the follicles and the oocytes can be damaged by heat stress even before ovulation.

The early embryo is also very sensitive to heat stress. In cattle, this has been shown in vivo (Putney et al., 1988a; Ealy et al., 1993) and in vitro (Edwards and Hansen, 1997; Rivera and Hansen, 2001). Heat stress also alters the embryonic environment by decreasing the synthesis of specific proteins from the endometrium (Malayer et al., 1988) and by reducing blood flow to the uterus (Roman-Ponce et al., 1978) and thereby altering perfusion of nutrients and hormones to the uterus. Heat stress induces a decrease in LH pulse frequency in early stages of the estrous cycle (Wise et al., 1988) , which might affect development of the corpus luteum. There is evidence that progesterone secretion is reduced in luteal cells collected from cows during summer as compared to winter (Wolfenson et al., 1993). Heat stress can also compromise maintenance of the corpus luteum since heat stress has been shown to increase prostaglandin $F_{2\alpha}$ synthesis from day 17 endometrium (Putney et al., 1988b; Malayer and Hansen, 1990b) and reduce secretion of interferon-τ from the conceptus (Putney et al., 1988b).

Among the mechanisms by which heat stress affects reproductive processes, much emphasis has been given to direct effects on the early embryo. This organism is very susceptible to maternal hyperthermia. Exposure of superovulated dairy heifers to environmental chambers at 42°C for 8 h from days 1 to 7 after estrus increased the proportion of abnormal and retarded embryos (Putney et al., 1988a). Some of this reduction in embryonic survival could involve direct disruption of elevated temperature in embryonic development while some could reflect alterations in maternal environment. Alliston and Ulberg (1961) conducted a reciprocal embryo transfer study in sheep to differentiate the relative importance of effects of heat stress on the embryo versus the dam. Donors and recipients were exposed to an ambient temperature of 21°C or 32°C in environmental chambers. Embryo transfers were performed about 3 days after onset of estrus. When embryo donor and recipient were maintained at 21°C, embryo survival was 56.5%. However, transfer of a non-heat stressed embryo to a heat stressed recipient reduced embryo survival to 24%. Only 9.5 % of embryos survived when a heat stressed embryo was transferred to a non-heat stressed recipient (Alliston and Ulberg, 1961). This result indicates that both the embryo and reproductive tract are sensitive to exposure to elevated temperature and the deleterious effects of heat stress are more pronounced in embryos.

Direct effects of elevated temperature on embryonic development have been shown in vitro. In cattle, exposure of zygotes (Rivera and Hansen, 2001), 2-cell (Edwards and Hansen, 1997; Rivera and Hansen, 2001; Al-Katanani and Hansen, 2002) 7and 4-8 cell stage embryos (Edwards and Hansen, 1997) to heat shock reduced development to the blastocyst stage. Heat shock also decreased protein synthetic capability in murine (Edwards et al., 1995) and bovine (Chandolia et al., 1999) embryos.

One approach to reduce the deleterious effects of elevated temperature on pregnancy rate per insemination is to make the embryo thermotolerant. To do so, it will be necessary to have a better understanding of the factors associated with embryonic resistance to elevated temperature. One characteristic of embryonic development is that embryos become more resistant to elevated temperature as they advance in development. This was demonstrated in vivo by exposing superovulated lactating Holstein cows to heat stress at different times after estrus (Ealy et al., 1993). Exposure to heat stress on day 1 after estrus reduced the proportion of embryos collected on day 8 that had developed to the blastocyst stage and were viable as determined by dye exclusion. In contrast, heat stress had no effect on embryos if heat stress was applied on days 3, 5 and 7 after estrus (Ealy et al., 1993). Moreover, heat shock of 41 or 42° C for 3 h decreased development of cultured 2-cell bovine embryos to $\geq$ 16 cell stage but had no effect on development of morula to the blastocyst stage (Ealy et al., 1995). Similarly, exposure of 2-cell and 4-8 cell bovine embryos to 42°C reduced development to the blastocyst stage but had no deleterious effects on embryos at the morula stage (Edwards and Hansen, 1997). It is likely that embryos acquire biochemical mechanisms for thermoprotection as they advance in development. Identification of such mechanisms could be used to prevent the deleterious effects of heat stress on early embryonic survival.

The most common strategy used to reduce the negative effects of elevated temperature on dairy cows has been to modify the cow's environment through the provision of shade (Roman-Ponce et al., 1977) and evaporative cooling systems (Her et al., 1988; Wolfenson et al., 1988; Ryan et al., 1992; Ealy et al., 1994). Nonetheless, seasonal variation in pregnancy rates persists in Florida dairies in which such housing has been adopted (Hansen and Aréchiga, 1999). The observation that embryos acquire resistance to elevated temperature as they advance in development (Ealy et al., 1993) supports the concept that transfer of day 7 embryos at the morula or blastocyst stage could bypass the deleterious effects of heat stress the oocyte and early embryo. This approach has been effective in several studies (Putney et al., 1989a; Ambrose et al., 1999; Drost et al., 1999; Al-Katanani et al., 2002a). Transfer of day 7 in vivo produced embryos collected from superovulated heifers to synchronized recipients increased pregnancy rates to 29.2% during summer in Florida as compared to 13.5% in cows artificially inseminated (Putney et al., 1989a). However, the high cost and labor involved with in vivo production of bovine embryos by superovulation prevents a more widespread use of this technique in large dairies. One alternative is to use embryos produced in vitro. Production of bovine embryos in vitro from oocytes collected from an abattoir is relatively inexpensive and allows embryo production on a large scale. One possibility would be to produce embryos in vitro during the winter, cryopreserve them, and then transfer embryos to recipients during the summer. However, there is evidence that in vitro produced embryos do not survive freezing as well as embryos produced in vivo (Drost et al., 1999). Moreover, the high proportion of undetected estrus periods during summer (Thatcher and Collier, 1986) limits the number of cows available for embryo transfer. This limitation can be overcome using timed embryo transfer protocols which do not rely on estrus detection. Recent studies showed that timed embryo transfer using fresh (nonfrozen) in-vitro produced embryos increased summer pregnancy rates as compared to timed artificial insemination. However, there was no differences in pregnancy rates between timed artificial insemination group and timed embryo transfer group using frozen in vitro produced embryos (Ambrose et al., 1999; Al-Katanani et al., 2002a). The low survival of frozen-thawed vitro produced embryos indicates that culture system for production of embryos in vitro need to be optimized to produce embryos with developmental competence similar to the ones produced in vivo.

The major focus of this dissertation is to identify determinants of embryonic resistance to elevated temperature. Identification of such factors may result in novel methods for protecting embryos from elevated temperature. The objective of the first series of studies was to determine whether heat shock induces apoptosis in bovine embryos in a developmentally-regulated manner and whether heat-induced apoptosis represents a mechanism for developmental acquisition of thermotolerance. TUNEL-positive embryos have been demonstrated in mouse (Brison and Schultz, 1997; Brison and Schultz, 1998; O'Neill, 1998), human (Yang et al., 1998; Hardy, 1999), and bovine (Byrne et al., 1999; Matwee et al., 2000; Neuber et al., 2000) species. Spontaneous apoptosis is a developmentally-regulated phenomenon that first occurs in bovine embryos at the 8-16 cell stage (Byrne et al., 1999; Matwee et al., 2000), coincident with the time of the major activation of the embryonic genome (Memili and First, 2000). Moreover, this form of cell death plays a role in mammalian development as a quality control mechanism to eliminate cells that are damaged, nonfunctional, abnormal or misplaced (Jacobson et al., 1997; Meier et al., 2000; Beere and Green, 2001). It is possible that heat shock induces activation of the apoptotic machinery in heat-damaged blastomeres to signal the removal of the affected cell without compromising the embryo as a whole while allowing overall development to continue. Perhaps, this specialized mechanism for cell death and removal is involved in developmental acquisition of resistance to heat shock and the lack of heat-induced apoptosis in early embryos may be one reason for the susceptibility of these embryos to elevated temperature.

The objective of the second series of studies was to determine whether embryos, lymphocytes and reproductive tract tissues from Brahman and Senepol cattle (thermotolerant breeds) are better able to survive and function after exposure to elevated temperature than embryos, lymphocytes and reproductive tissues from Holstein and Angus cattle (thermosensitive breeds). Detrimental effects of heat stress on fertility are less pronounced in heat-tolerant breeds, in large part because of differences in thermoregulation (Adeyemo et al., 1979; Bennett et al., 1985; Hammond et al., 1996; Hammond et al., 1998; Gaughan et al., 1999). However, there is some evidence that cells from heat-tolerant breeds are less adversely compromised by elevated temperature than cells from heat-sensitive breeds (Malayer and Hansen, 1990a; Kamwanja et al., 1994). If such genetic differences extend to the embryo, identification of genes controlling differences in thermoregulation might lead to genetic approaches for producing an embryo with increased resistance to heat shock.

The last approach tested for increasing embryonic resistance to heat shock was to manipulate antioxidant status. In studies performed in Mexico, intramuscular injection with vitamin E and selenium 3 weeks before expected calving or 30 days postpartum increased pregnancy rates, reduced the number of services per conception and decreased the interval from calving to conception (Aréchiga et al., 1994b; Aréchiga et al., 1998b). Moreover, culture of murine embryos with vitamin E reduced the negative effects of heat shock on viability (Aréchiga et al., 1994a). Given that the deleterious effects of elevated temperature is associated with increased production of free radicals (Loven, 1998), it is possible that the beneficial effects of supplemental antioxidants such as vitamin E and selenium on fertility are greater in cows inseminated during summer as compared to cows inseminated during winter.

CHAPTER 2
LITERATURE REVIEW

Characteristic Effects of Heat Shock on Embryonic Development

Maternal hyperthermia in females exposed to heat stress during preimplantation development reduces embryo survival (Dutt, 1963; Tompkins et al., 1967; Dunlap and Vincent, 1971; Putney et al., 1988a; Ealy et al., 1993). Exposure of superovulated dairy heifers to controlled heat stress of 42°C for 8 h from days 1 to 7 after estrus increased the proportion of abnormal and retarded embryos (Putney et al., 1988a). Similarly, Dunlap and Vincent (1971) showed that conception rates in beef heifers were 48% in control animals maintained at 21°C versus 0% in heifers exposed to 32°C for 72 h after breeding . In part, this embryonic loss occurs because preimplantation embryos have limited ability to cope with disruptions in the thermal environment. In particular, early cleavage stage embryos are very sensitive to elevated temperature. Direct effects of elevated temperature (i.e., heat shock) on embryonic development has been observed in vitro. Exposure of 1-cell rabbit embryos to heat shock of 40° C for 6 h decreased postimplantation development when embryos were transferred to recipients (Alliston et al., 1965). In mouse embryos, heat shock disrupted embryonic viability as determined by live/dead staining (DAPI) (Aréchiga et al., 1994a; Ealy and Hansen, 1994; Aréchiga et al., 1995) and subsequent embryonic development (Aréchiga et al., 1994a; Ealy and Hansen, 1994; Aréchiga et al., 1995). In bovine embryos, heat shock of 41°C during the 8 h period of in vitro fertilization reduced cleavage rate and development to the blastocyst stage (Rivera and Hansen, 2001). Similarly, heat shock of bovine zygotes (Rivera and Hansen, 2001), 2-cell embryos (Edwards and Hansen, 1997; Rivera and Hansen, 2001; Al-Katanani and Hansen, 2002) and 4-8 cell stage embryos (Edwards and Hansen, 1997) reduced development to the blastocyst stage. For example, development to the blastocyst stage was reduced from 33% in 2-cell embryos exposed to 38.5° C to 5 % in embryos subjected to 41°C for 9 h (Rivera and Hansen, 2001). This result indicates that early embryos may lack some protective mechanisms or molecules that are involved in protection against the deleterious effects of elevated temperature.

In several species, the deleterious effects of elevated temperature decrease as embryos advance in development. This has been shown in vivo in sheep (Dutt, 1963), pigs (Tompkins et al., 1967), cattle (Ealy et al., 1993) and rabbits (Wolfenson and Blum, 1988). Ealy et al. (1993) conducted a study in which superovulated lactating Holstein cows were exposed to no-shade heat stress environment on days 1, 3, 5 or 7 after estrus or not heat stressed. On day 8, embryos were flushed from the uterus and evaluated for stage of development and viability. The percentages of embryos developing to the blastocyst stage were 60.6% for control and 37.4, 54.8, 62.6 and 64.5% for embryos from cows heat stressed on days 1, 3, 5, and 7, respectively. Similarly, the percentages of live embryos were 70.2% for control and 55.0, 68.0, 65.0 and 88.9 for embryos from cows heat stressed on days 1, 3, 5, and 7, respectively. Thus, heat stress reduced development to the blastocyst stage and embryonic viability on day 1 but not days 3, 5 or 7 after estrus. This developmental acquisition of thermotolerance has also been shown in vitro. Exposure of 2-cell bovine embryos to 41 or 42° C for 3 h decreased development to ≥ 16 cell stage (Ealy et al., 1995) while this same heat shock had no effect on development of morula to the blastocyst stage. When bovine embryos at 2-cell and 4-8 cell stage were exposed to 42°C, heat shock reduced development to the blastocyst stage but had no deleterious effects on embryos at the morula stage (Edwards and Hansen, 1997). Similarly, exposure of 2-cell bovine embryos to heat shock of 41°C caused a time-dependent decrease in the percentage of embryos that developed to the blastocyst stage (Kríninger III et al., 2002) while this same heat shock had no effect on development of embryos ≥ 16 cell to the blastocyst stage. It is possible the high number of cells present in a morula stage embryo allows survival even after the loss of a fraction of its cells whereas if a 2-cell embryo loses one blastomere after heat shock it would have only one blastomere left to form a viable embryo. Another possibility is that embryos acquire one or more thermoprotective responses as embryonic development proceeds.

Mechanisms for Disruption in Preimplantation Embryonic Development by Heat Shock

The mechanisms by which heat shock affects embryonic function are only partially identified. Exposure of mouse embryos to elevated temperature resulted in retarded development and reduced ability to incorporate [^{3}H]uridine (Bellve, 1972; Bellve, 1976). Heat shock also decreased protein synthetic capability in embryos (Hahnel et al., 1986; Edwards et al., 1995; Chandolia et al., 1999). Exposure of mouse preimplantation embryos to 43° C for 1 h caused a 75-85% decrease in synthesis of intracellular proteins at all stages examined (Hahnel et al., 1986). Similarly, exposure of mouse expanded blastocysts to 43°C reduced protein synthesis as measured by incorporation of ^{35}S-labeled amino acids into TCA-precipitable protein (Edwards et al., 1995). A less severe heat shock of 42°C for 1.33 h also reduced the protein synthetic capability of 2-cell bovine embryos (Chandolia et al., 1999). Moreover, exposure to 40°

C reduced protein synthesis in mouse morula (Aréchiga et al., 1995) but not in expanded blastocysts (Edwards et al., 1995) indicating that embryos at this stage are more resistant to heat shock.

Involvement of free radicals in effects of heat shock on embryos is indicated by experiments examining the relationship between the antioxidant glutathione (GSH) and embryonic resistance to heat shock. Intracellular levels of GSH are reduced by heat shock. The GSH content in mouse morulae cultured at 37°C was 0.068 pmol/embryo versus 0.035 pmol/embryo for embryos subjected to 41°C for 1 h (Aréchiga et al., 1995), suggesting that GSH is involved in removal of free radicals generated by heat shock. Culture of embryos in the presence of S-adenosyl-l-methionine (SAM), an inducer of GSH synthesis, increased viability (dye-exclusion) of heat-shocked morulae from 25% in embryos exposed to 43°C to 79% in embryos exposed to 43°C in the presence of SAM and increased development of heat shocked embryos to the blastocyst stage from 38% to 51% (Aréchiga et al., 1995). Similarly, direct addition of GSH to culture medium of mouse embryos reduced the deleterious effects of heat shock on viability (Aréchiga et al., 1994a; Aréchiga et al., 1995). However, GSH didn't prevent the reduction in development to the blastocyst stage caused by heat shock. Thus, GSH can partially protect embryos from heat shock effects.

Ultrastructural alterations in 2-cell bovine embryos exposed to heat shock has recently been reported (Rivera and Hansen, 2002). Heat shock at both 41 and 43°C displaced organelles to the center of the blastomere suggesting cytoskeleton rearrangement. Moreover, the percentage of swollen mitochondria was increased by both 41 and 43° C. Separation between the two membranes comprising the nuclear envelope was observed in embryos exposed to 43° C but not in embryos exposed to the more physiological temperature of 41°C.

Mechanisms Involved in Developmental Acquisition of Embryonic Resistance to Heat Shock

As described earlier, embryos become more resistant to elevated temperature as they advance in development. The timing of when embryos become more resistant to heat shock (between the 2-8 cell stage and the morula stage) is temporally coincident with when the embryo first undergoes large-scale embryonic transcription (between the 8-16 cell stage (Memili and First, 2000). This section will address the relationship between activation of the embryonic genome and the possible role of heat shock proteins in developmental acquisition of thermotolerance.

Embryonic Genome Activation

Embryonic genome activation refers to the developmental transition from maternal to embryonic genetic control of development. As embryonic development proceeds and maternally inherited RNAs and proteins are degraded, embryonic development becomes dependent on expression of embryonic genes (Schultz et al., 1995). Depending on the species, embryonic genome activation has been shown to occur at the 8- to 16-cell stage in the cow (Kopecny et al., 1989; Barnes and First, 1991), sheep (Crosby et al., 1988) and rabbit (Brunet-Simon et al., 2001), 4- to -8-cell stage in humans (Braude et al., 1988), 4-cell stage in the pig (Kopecny, 1989) and 2-cell stage in the mouse (Flach et al., 1982). The lack of full transcriptional activity observed in the early embryo limits the embryo's ability to adjust to changes on its environment. Therefore, it is possible that the increased sensitivity of the early embryo to elevated temperature (Ealy et al., 1992; Ealy et al., 1995; Edwards and Hansen, 1997; Rivera and Hansen, 2001) is related to the fact that the embryonic genome is suppressed mostly during early cleavage stages.

In bovine embryos, the major onset of genome activation occurs at the 8-16 cell stage. Bovine embryos cultured in the presence of the RNA polymerase II inhibitor (α-amanitin) arrested in development at the 8- to 16-cell stage while control embryos progressed to the morula and blastocyst stage (Jones and First, 1990) indicating the requirement for embryonic transcripts at this stage. However, α-amanitin sensitive [^{3}H] uridine incorporation was observed as early as the 1- and 2-cell stage (Memili and First, 1999). This result demonstrate that bovine zygotes and 2-cell embryos are transcriptionally competent.

There is evidence that DNA replication and chromatin structure play a role in initiation of transcription in bovine embryos. The highly condensed chromatin is formed by nucleosomes, which consist of four core histones wrapped around 160 base pairs of DNA. The N-terminal domain of histones is formed by a positively-charged lysine tail that binds to the negatively-charged DNA to form a compact structure (Hecht et al., 1995). This compact chromatin structure represses transcription by blocking access of transcription factors to their DNA sequences. In contrast, a loose chromatin structure is associated with increased transcriptional activity. Post-translational histone modification such as acetylation can disrupt interaction between histones and DNA resulting in a looser chromatin structure and enhancement of transcriptional activity. In contrast, deacetylation of histones causes transcriptional silencing (Wolffe and Prus, 1996).

Culture of 1- and 2-cell bovine embryos with [^{3}H]uridine in the presence of an inhibitor of DNA replication (aphidicolin) reduced [^{3}H]uridine incorporation and uptake

(Memili and First, 1999). Thus, blocking DNA replication reduced transcriptional activity in bovine embryos. In addition, culture of 1- and 2-cell bovine embryos in the presence of an inhibitor of histone deacetylase (tricostatin) increased [^{3}H]uridine incorporation and uptake (Memili and First, 1999). It is likely that histone hyperacetylation disrupted the nucleosomes and created an opportunity for transcription.

In the mouse, there is a minor round of transcription at the 1-cell stage, but the major burst of activation occurs at the 2-cell stage. Mouse embryos treated with α-amanitin arrest in development at the 2-cell stage (Golbus et al., 1973; Flach et al., 1982). Incorporation of BrUTP by mouse embryos starts at the 1-cell stage and gradually increases (Aoki et al., 1997). Even though minor transcriptional activity starts at the 1-cell stage, translation of zygotic transcripts does not start until the 2-cell stage (Aoki et al., 1997; Davis and Schultz, 1997). Protein synthesis at the 2-cell stage is dependent upon transcription because synthesis of polypeptides in the 2-cell mouse embryo was sensitive to α-amanitin (Davis and Schultz, 1997). Similarly, the DNA replication inhibitor aphidicolin blocked synthesis of some polypeptides (Davis and Schultz, 1997). Thus, zygotic activation is accompanied by the translation of both replication-dependent and replication-independent polypeptides (Davis and Schultz, 1997). There is additional evidence that, as is the case for bovine embryos, chromatin maturation is implicated in transcription initiation in the mouse. Inhibiting the first round of DNA replication did not prevent transcription at the 1-cell stage but inhibited BrUTP incorporation by 35%. Moreover, inhibition of histone deacetylase with trapoxin caused enhanced incorporation of BrUTP by 2 cell embryos (Aoki et al., 1997).

In the mouse embryo, initiation of transcription also may be regulated in a time-dependent manner rather than stage of development. For instance, the expression of the transcription-requiring complex in the mouse occurs at the same time in control embryos or embryos cultured with an inhibitor of cytokinesis (cytochalasin D) or an inhibitor of DNA synthesis (aphidicolin) (Schultz, 1993). Thus, the zygotic clock might act by modulating the function of transcriptional factors or delaying embryonic genome activation until chromatin is remodeled.

Heat Shock Proteins

All living organisms have evolved adaptive mechanisms that allow a rapid response to exposure to elevated temperature. This cellular response, known as the heat shock response, switches off synthesis of most proteins and initiates large scale synthesis of a set of proteins called heat shock proteins (HSPs) (Lindquist and Craig, 1988). Heat shock proteins are divided into six major families: HSP110, HSP90, HSP70, HSP60, HSP27 and ubiquitin (Nover and Scharf, 1991). In addition to heat shock, other cellular stresses including anoxia (Sciandra et al., 1984), oxidizing agents (Spitz et al., 1987), amino acid analogs (Kelley and Schlesinger, 1978), ethanol (Li and Werb, 1982), glucose deprivation (Sciandra et al., 1984), high pH (Whelan and Hightower, 1985), bacterial and viral infections (Rose and Khandjiam, 1985) can lead to activation of HSP synthesis.

The HSP70 family is the most studied group of heat shock proteins. Mammalian HSP70 family consists of two cytosolic isoforms, a constitutive variant (HSC70 or HSP73) and a highly inducible isoform (HSP70 or HSP72) (Welch et al., 1983), a mitochondrial isoform (mtp70 or Grp75) (Mizzen et al., 1989) and an isoform in the endoplasmic reticulum (Bip or Grp78) (Munro and Pelham, 1986). All these proteins are

ATP-binding proteins and have weak ATPase activity which can be stimulated upon binding to unfolded proteins and synthetic polypeptides (Rothman, 1989).

Under physiological conditions, HSPs act as molecular chaperones. As a newly synthesized polypeptide emerges from a ribosome, it is bound to a form of HSP70 in the cytosol which prevents the premature folding of a growing polypeptide. Once polypeptide synthesis is complete, HSP70 escorts and transfers the new molecule to HSP60 to ensure proper folding and assembly. Many of the proteins synthesized in free ribosomes are destined to reside in organelles such as the endoplasmic reticulum, mitochondria and nucleus. Heat shock protein 70 mediates this transport of proteins to these organelles (Chirico et al., 1988; Craig et al., 1993). In the case of proteins destined for secretion, a form of HSP70 called Bip transports nascent polypeptides across the membranes of the endoplasmic reticulum (Brodsky and Schekman, 1994). There is also evidence that HSPs interact with receptors for steroid hormones. Studies indicated that HSP70 associates with steroid hormone receptors to maintain these transcription factor inactive and unable to bind DNA (Pratt et al., 1992). Binding by a specific ligand causes dissociation of HSP70 in an ATP-dependent manner (Kost et al., 1989; Smith and Toff, 1993) to allow the receptor to bind DNA (Pratt et al., 1992). Addition of monoclonal antibody against HSP70 to reticulocyte lysates prevented HSP90 from binding to the steroid receptor complex (Smith and Toff, 1993). Thus, HSP70 may play a role stabilizing steroid receptor and assist HSP90 to prevent DNA binding of non-steroid bound receptors

Many cellular stresses compromise protein structural integrity by exposing hidden hydrophobic domains. Under conditions such as elevated temperature, HSPs bind and stabilize damaged proteins in a partially unfolded conformation to prevent protein aggregation in the cytosol and create an opportunity for refolding or degradation (Hartl and Martin, 1992; Becker and Craig, 1994). Moreover, stress stimuli causes HSP70 migration and accumulation in the nucleolus where they associate with partially assembled ribosomes to protect the nucleus (Welch and Feramisco, 1984). Heat shock proteins can also protect cells from heat-induced apoptosis. Both HSP27 (Bruey et al., 2000) and HSP70 (Wei et al., 1995; Mosser et al., 1997; Mosser et al., 2000) have been shown to block apoptosis.

Heat shock protein 70 has been implicated in induced thermotolerance. This phenomenon is defined as the ability of cells to withstand severe heat shock after exposure to a mild heat shock (Nover and Scharf, 1991). Indeed, induction of thermotolerance in HeLa cells is associated with accumulation of HSP70 (Mizzen and Welch, 1988). Microinjection of HSP70 antibody into fibroblasts prevented heat-induced translocation of HSP70 to the nucleus and blocked induction of thermotolerance (Riabowol et al., 1988). In addition, administration of constitutive HSP70 mRNA can modulate fibroblast responses to heat shock inducing thermotolerance (Li et al., 1991). Targeted disruption of DNA binding activity of the transcription factor for heat shock proteins (HSF-1) in mouse embryonic fibroblasts blocked thermotolerance and prevented protection against heat-induced apoptosis (McMillan et al., 1998). Thus, previous exposure to a mild heat shock can induce accumulation of HSP70 to make cells more resistant to a subsequent more severe heat shock.

Synthesis of HSP70 in mammalian cells is an autoregulated process that involves the heat shock transcription factors (HSFs) (Morimoto et al., 1992; Pirkkala et al., 2001).

In vertebrates, HSF-1 is involved in stress-inducible transcription of HSPs while HSF-2 is involved in transcription of the constitutive proteins refractory to classical stress stimuli (Pirkkala et al., 2001). In unstressed cells, interactions with HSP70 causes HSF-1 to exist in a monomeric form without DNA binding activity. Heat shock leads to accumulation of denatured and misfolded proteins and the available HSP70 then dissociates from HSF-1. The free HSF-1 is then phosphorylated by protein kinases such as protein kinase C and trimerizes. Trimers translocate to the nucleus and bind heat shock elements on the promoter region of HSP gene leading to transcription and translation of new HSPs. These newly synthesized HSPs return to the cytosol and binds HSF-1 to downregulate its own synthesis (Morimoto et al., 1992; Pirkkala et al., 2001).

Role of Heat Shock Proteins in Preimplantation Embryos and Induced Thermotolerance

Heat shock proteins plays an important role in preimplantation embryonic development. Inhibition of mouse HSPs with antisense oligonucleotides complementary to the mRNAs of hsp70-1 and hsp70-3 reduced development to the blastocyst stage from 90% in control embryos to 30% (5 μM antisense oligonucleotides) or 0% (10 μM antisense oligonucleotides) in embryos receiving antisense treatments (Dix et al., 1998). Another approach used to block embryonic HSPs has been to culture embryos with specific antibodies. In mouse embryos, monoclonal antibodies specific for HSP60, HSP70 or HSP90 reduced subsequent development (Neuer et al., 1998). The percentage of hatched blastocysts decreased from 68% in control embryos to 29% in embryos treated with anti-HSP60, 28 % with anti-HSP70, and 57% when embryos were cultured with anti-HSP90 (Neuer et al., 1998). When bovine embryos were cultured with antibody specific to HSP70 from days 3 to day 9 post-insemination there was a decrease in development to the blastocyst stage (16.1% in control versus 7.7% in anti-HSP70-treated embryos) and an increase the percentage of apoptotic cells in blastocysts (5.7% in control versus 8.0% in anti-HSP70-treated embryos) (Matwee et al., 2001). In addition, culture of 2-cell bovine embryos under 38.5 or 41°C in the presence of anti-HSP70i caused a decrease in the proportion of embryos that developed to the blastocyst stage regardless of culture temperature (Al-Katanani and Hansen, 2002). However, addition of anti-HSP70 did not increase susceptibility of embryos to the deleterious effects of heat shock

The mechanism by which HSP antibody affects embryonic development is not clear. It is possible that HSP70 antibody crosses the cell membrane and neutralizes intracellular HSP70. Indeed, the idea that antibodies are unable to penetrate living cells has been challenged. For example, IgG anti-ribonucleoprotein and IgG anti-DNA can penetrate the membrane of epithelial and fibroblast cells (Alarcón-Segovia et al., 1996). Even though HSPs are mainly intracellular proteins, some members of HSP70 family were localized on the cell surface of several cell types (Multhoff et al., 1997; Botzler et al., 1998). Thus, it is also possible that HSP70 antibody neutralizes membrane bound forms of HSP70.

In the mouse embryo, the hsp70 gene is first expressed at the 2-cell stage coincident with the time of major genome activation (Bensaude et al., 1983; Manejwala et al., 1991; Christians et al., 1995). In fact, hsp70 transcripts are one of the first produced during gene activation in mouse embryos. In contrast to this early spontaneous expression of the hsp70 gene, initial studies in mice indicated that heat-induced synthesis of HSP70 first occurred at the morula and blastocyst stage, coincident to the time in development in which embryos were more resistant to heat shock (Morange et al., 1984;

Muller et al., 1985; Hahnel et al., 1986; Heikkila et al., 1986). A more recent study indicated that exposure of mouse embryos at the 8-cell stage to 40°C enhanced synthesis of constitutive HSC70 proteins and induced synthesis of the inducible HSP68 (Edwards et al., 1995). It is possible that some of the earlier studies did not detect heat-inducible synthesis of HSP70 in early embryos because the high temperature used (43°C) was so severe it blocked the heat shock response.

In bovine embryos, the idea that the embryonic genome is transcriptionally inactive during early stage of development led to the hypothesis that early embryos, were sensitive to heat shock because they were unable to synthesize HSPs. However, several experiments have demonstrated the contrary. Exposure of 2-cell bovine embryos to heat shock induced the heat-inducible HSP68 (Edwards and Hansen, 1996; Edwards et al., 1997). The transcription inhibitor α-amanitin which prevents binding of RNA polymerase II to the promoter (Lindell et al., 1970) blocked heat-induced increased of HSP68 in embryos at the 4-cell stage or greater but not in embryos at the 2-cell stage (Edwards et al., 1997). However, a more recent study using RT-PCR showed that the transcription inhibitors 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, which blocks activity of RNA polymerase II at the level of transcription elongation (Yamaguchi et al., 1998), and actinomycin D which binds DNA and prevents RNA elongation (Sobel, 1985) reduced expression of HSP70 transcripts in bovine embryos at the 2- and 4-cell stage (Chandolia et al., 1999). These results indicate that heat shock is upregulating HSP70 transcription. Thus, bovine embryos as early as the 2-cell stage are able to respond to heat-shock by undergoing transcription and translation of HSP70.

Acquisition of resistance to lethal heat shock after exposure to a mild heat shock has been demonstrated to occur in the mouse (Muller et al., 1985; Ealy and Hansen, 1994; Aréchiga et al., 1995; Aréchiga and Hansen, 1998) and bovine (Ealy and Hansen, 1994; Jyh-Cherng et al., 1999; Al-Katanani and Hansen, 2002) preimplantation embryos in a developmentally-regulated manner. In mice, induced thermotolerance was not seen at the 2-cell stage but was observed in 8-cell embryos, morulae and blastocysts (Muller et al., 1985; Ealy and Hansen, 1994; Aréchiga et al., 1995; Aréchiga and Hansen, 1998). In contrast, induced thermotolerance in bovine embryos has only been observed at the blastocyst stage of development (Ealy and Hansen, 1994). Exposure of day 3, 8-cell stage bovine embryos to a mild heat shock of 40.5°C for 1 h did not prevent the reduction in development caused by a lethal heat shock of 43°C for 1 h (Jyh-Cherng et al., 1999). Moreover, bovine 2-cell embryos which express (Chandolia et al., 1999) and synthesize (Edwards and Hansen, 1996; Edwards et al., 1997) HSP70 in response to heat shock were unable to undergo induced thermotolerance (Al-Katanani and Hansen, 2002). Therefore, acquisition of the ability to undergo heat-induced HSP70 synthesis is not sufficient for developmental acquisition of thermotolerance.

Apoptosis as a Determinant of Embryonic Resistance to Heat Shock

General Features of Apoptosis

Apoptosis is an active process of cellular self destruction or suicide. Apoptosis can be considered as complementary to mitosis and together these two processes regulate development, growth, involution and maintenance of tissues and organisms.

According to Clarke and Clarke (1996) , the recognition of cell death as a naturally occurring phenomenon was first described by Vogt in 1842 during studies with amphibian metamorphosis. Programmed cell death was later demonstrated to occur in tissues of both invertebrates and vertebrates (Glucksmann, 1951; Clarke and Clarke, 1996). Originally, the term "programmed cell death" was used to describe events of cellular death that occurred in a spontaneous, orchestrated and predictable manner throughout animal development (Lockshin and Williams, 1964). It was only in 1972 that Kerr, Wyllie and Currie established the similarities among many models of cell death and described a conserved set of morphological features observed in several cell types during physiological episodes of cell death in mammals. This programmed cell death was named apoptosis (Kerr et al., 1972). The word apoptosis comes from the Greek language where "*apo*" means from and "*ptosis*" means to fall. Thus, apoptosis means "to fall away from." Cell death observed during normal animal development and some pathological conditions was differentiated from the accidental cell death that occurred in consequence of acute lesions (Kerr et al., 1972).

Landmark genetic studies in the nematode *Caenorhabditis elegans* conclusively established the molecular regulation of the process by which cells cause their own suicide. These studies have identified major genes involved in apoptosis (ced-3, ced-4 and ced-9) (Horvitz et al., 1982; Ellis and Horvitz, 1986) and led to the discovery of their mammalian homologous (Yuan et al., 1993; Hengartner and Hortvitz, 1994). The *C. elegans* gene, ced-3, is homologous to the mammalian interleukin-1β-converting enzyme (ICE) or caspase-1. Fourteen caspases have been identified and several of them play a major role in the disassembly of cytoplasmic and nuclear structures during apoptosis. The Bcl-2 family of proteins, homologous to ced-9, are regulatory molecules that act as anti- or pro-apoptosis depending on their dimerization pattern and cellular context. The ced-4 gene is homologous to the mammalian Apaf-1 (apoptotic protease activating factor-1). Apaf-1 plays a role by binding to cytosolic cytochrome c in order to modulate the mitochondrial pathway of caspase activation (Metzstein et al., 1998).

The fact that cell death is an important process that contributes to normal development and disease has now become clear. Apoptosis has been proposed as a major regulatory event observed during embryonic development, establishment of immune self-tolerance, immune effector cell killing and regulation of cell viability by hormones and growth factors (Zakeri and Lockshin, 2002). Alteration of the cell death program may contribute to cancer, viral pathogenesis, acute neurological injuries, neurodegenerative diseases, cardiovascular diseases, autoimmune diseases and acquired immunodeficiency syndrome (Zimmermann et al., 2001).

A typical feature of animal development is the excessive production of cells that are later removed to ensure the establishment and maintenance of tissue architecture. Thus, the ability to delete cells is as important for an organism as the ability to proliferate and differentiate. Some of the functions of apoptosis in animal development are: 1) to act as a quality-control mechanism to eliminate cells that are abnormal, misplaced, nonfunctional or dangerous for the organism as a whole; 2) prevent ectopic cellular proliferation; 3) sculpt parts of the body as exemplified by removal of cells between the digits in vertebrates and 4) delete cells that are no longer needed and adjust cell numbers (Jacobson et al., 1997).

Morphological and Biochemical Features of Apoptosis and Necrosis

Apoptosis and necrosis are the most widely described forms of cell death and they can be distinguished based on their morphological and biochemical characteristics. Much of the morphological characterization of apoptosis has been performed using light and electron microscopy. Morphologically, apoptosis involves a series of consecutive phases that affect the nucleus and cytoplasm of a cell (Wyllie et al., 1980). Initially, the cell shrinks and becomes denser (Kerr, 1971). The chromatin aggregates and condenses to form large compact granular masses applied against the nuclear membrane. The chromatin has a curved appearance commonly described as half-moon-, horse-shoe-, sickle-, lancet-, and ship-like (Majno and Joris, 1995). The nuclear envelope remains morphologically intact but the nuclear pores are redistributed and accumulate between the condensed chromatin (Earnshaw, 1995). The nucleus progressively condenses and the nuclear outline becomes first convoluted, then grossly indented and then broken up (karyorhexis) to generate nuclear fragments containing condensed chromatin (Wyllie et al., 1980). The nucleolus is usually considered very resistant to apoptosis (Columbaro et al., 1998; Martelli et al., 1999). In some apoptotic models, however, there is separation and condensation of the granular and fibrillar nucleolar components (Martelli et al., 2000).

Parallel to these nuclear changes, the cytoplasm starts to condense, the cell rounds up, the plasma membrane becomes indented and the microvilli disappear (Kondo et al., 1997). Organelles such as mitochondria, endoplasmic reticulum and lysosomes can maintain an intact shape and structure during the initial stages of apoptosis (Kerr et al., 1972). However, dilatation of the endoplasmic reticulum (Pollard et al., 1987; Ludewig et al., 1995), detachment of ribosomes from the rough endoplasmic reticulum together with ribosome aggregation (Ferguson and Anderson, 1981), and condensation of the mitochondria (Kluck et al., 1999) can be observed as late apoptotic events.

One of the most striking alterations observed during apoptosis is the change in cell shape due to reorganization of the outer borders of the cell. The affected cell loses contact with its neighboring cells and extracellular matrix. As the cytoplasm condenses, the organelles become compressed and there is the formation of cytoplasmic vacuoles. The dying cells emit several protrusions from the cell surface called buds (Kerr and Harmon, 1991). Finally, the whole cell fragments into membrane-bound apoptotic bodies of roughly spherical or ovoid shape (Kerr et al., 1972; Mills et al., 1998). Most of the time these membrane-bound vesicles are phagocytosed by neighboring cells and there is no exudative inflammation. In some cases, the apoptotic bodies are dispersed in the intercellular tissue spaces and extruded into an adjacent lumen. In this case, the apoptotic bodies undergo spontaneous degeneration typical of necrosis (Wyllie et al., 1980).

The appearance of cells undergoing apoptosis is distinct from that caused by the other major type of cell death, necrosis. Necrosis or accidental cell death results from injury, toxic agents or ischemia, and affects large number of contiguous cells (Wyllie et al., 1980). At the early stages, there is loss of plasma membrane integrity followed by cellular swelling and marginal clumping of the chromatin. In the cytoplasm, there is dilation of the endoplasmic reticulum, dissolution of ribosomes and lysosomes, swelling of the mitochondrial matrix, and rupture of nuclear, organelle, and plasma membranes. Membrane rupture is followed by the release of intracellular content and debris which triggers an exudative inflammation (Wyllie et al., 1980).

Apoptosis and necrosis have long been described as two distinct modes of cell death, with each one carrying specific morphological and biochemical markers. There is now some evidence, however, that a dying cell can share some characteristics of both apoptosis and necrosis in a state called aponecrosis (Formigli et al., 2000). It is also evident that there are common mechanisms involved in these two forms of cellular death. Depending on the intensity and severity of the damage, a cell can undergo death by apoptosis, aponecrosis or necrosis. In fibroblasts treated with a metabolic poison (antimycin A) there was induction of apoptosis, aponecrosis and then necrosis as the concentration of the poison was increased (Formigli et al., 2000). Other studies have also demonstrated that low concentrations of a toxicant would trigger apoptosis while a high concentration of the same toxicant would trigger necrosis (Lennon et al., 1991). Likewise, low to moderate concentrations of oxidants leads to DNA fragmentation while high concentrations induce necrosis without DNA fragmentation (McConkey et al., 1988). One important determinant for the mode of cell death is the energy status of the cell. The level of ATP can act as a switch between apoptosis and necrosis (Eguchi et al., 1997; Leist et al., 1997). There is evidence that necrosis is preceded by a dramatic drop in ATP levels ranging between 50-70% (Leist et al., 1997). Moreover, caspase activation and DNA fragmentation were blocked by ATP depletion (Eguchi et al., 1997; Leist et al., 1997). Changes in intracellular calcium concentrations have also been involved in the pattern of cell death. Low to moderate (200-400 nM) Ca^{2+} concentrations are associated with apoptosis (McConkey and Orrenius, 1996) while massive Ca^{2+} influx (above 1 μM) causes necrosis due to mitochondrial Ca^{2+} overload, mitochondrial membrane potential collapse and ATP depletion (Richter, 1993). In some apoptotic models, inhibition of caspases prevented apoptosis and resulted in cell death by necrosis (Hirsch et al., 1997).

In recent years, the interest in identifying characteristic features of apoptosis has shifted from morphological to biochemical and molecular markers. Mitochondrial membrane permeabilization (Green and Kroemer, 1998) is an early event during the apoptotic program. It differentially affects the outer and the inner mitochondrial membrane. The outer mitochondrial membrane becomes permeable to proteins, while the inner membrane continues to retain proteins from the mitochondrial matrix (Patterson et al., 2000). Therefore, only proteins from the intermembrane space are released from mitochondria. Moreover, the decrease in mitochondrial membrane potential seems to be irreversible and regulated by the Bcl-2 family of proteins (Hirsch et al., 1997; Deshmukh et al., 2000; Goldstein et al., 2000). Another early feature of apoptosis is the loss of membrane asymmetry and externalization of phosphatedylserine to the outer leaflet of the plasma membrane. In healthy cells, phosphatedylserine is usually located in the inner leaflet of the plasma membrane. Induction of apoptosis causes exposure of phosphatedylserine which acts as a signal leading to phagocytosis of the apoptotic cell by neighboring cells (Martin et al., 1995). At the nucleus, the most well known biochemical change observed during cell death by apoptosis is the caspase-mediated cleavage of the linker DNA between nucleosomes to generate internucleosomal DNA fragments (Wyllie et al., 1980; Arends and Wyllie, 1991). Agarose gel electrophoresis of such DNA shows a characteristic ladder pattern with bands at 180-200 bp in size. In contrast, DNA from necrotic cells is cleaved at random and forms a smear on agarose gels.

The nuclear matrix is composed of nonhistone proteins and RNA. It forms a fibrogranular network through the nucleus that connects with the peripheral lamina (Pederson, 2000). It is now apparent that early proteolysis of key nuclear matrix proteins may facilitate the breakdown of the nuclear matrix and the entire nucleus (Robertson et al., 2000). Nuclear lamins, proteins that are normally attached to the inner side of the nuclear envelope, were among the earliest proteins identified as caspase substrates (Moir and Goldman, 1993). Expression of uncleavable mutants of either lamin A and lamin B prevented or delayed changes in nuclear morphology (Rao et al., 1996), suggesting that lamins may participate to maintain nuclear integrity.

Bcl-2 Related Proteins

The *C. elegans* protein CED-9 is homologous to Bcl-2 in mammals (Vaux et al., 1992). The Bcl-2 protein family is formed by anti-apoptotic (Bcl-2, Bcl-xL, Bcl-w, Bfl-1, Brag-1, Mcl-1 and Al) and pro-apoptotic (Bax, Bak, Bcl-xs, Bad, Bid, Bik, Hrk and Bok) proteins. The ratio of these two types of proteins determine in large part whether the cell will be committed to death following an apoptosis-inducing stimulus (Motyl, 1999). Members of this family of proteins are characterized by four structural domains: BH-1, BH-2, BH-3 and BH-4. The Bcl-2 homology domains (BH) (Oltvai et al., 1993; Yin et al., 1994) correspond to α-helical segments of the protein that are involved in protein-protein interactions (Kelekar and Thompson, 1998). Mutagenesis studies indicated that BH-1, BH-2 and BH-3 domains play a role in homo- and heterodimerization of Bcl-2 proteins (Yin et al., 1994; Chittenden et al., 1995) while the BH-4 domain is involved in interaction with proteins outside the Bcl-2 family (Wang et al., 1996; Shibasaki et al., 1997). Several pro-apoptotic proteins (Bax, Bak, Bik and Bad) lack the BH-4 domain (Basu and Haldar, 1998).

Bax was the first Bcl-2 related protein identified. Functional assays demonstrated that Bax co-immunopreciptates with Bcl-2 in vitro and suppresses the ability of Bcl-2 to block apoptosis (Oltvai et al., 1993). The ability to form homo- and heterodimers and to function as integral membrane proteins are important features of these proteins. In viable cells, Bax monomer is located either in the cytosol or loosely attached to the membranes. After a death signal, Bax translocates to the mitochondria and inserts in the membrane as an integral protein and forms a homodimer (Gross et al., 1998). There is evidence that Bax homodimers increase permeabilization of the outer mitochondrial membrane resulting in the release of apoptogenic proteins. For example, Bax (Rosse et al., 1998) and Bid (Luo et al., 1998) promote cytochrome c release from the mitochondria. Disruption of the BH3 domain of Bax impairs its ability to form a homodimer and prevents cell death. Excess of Bax results in Bax-Bax homodimers and counteracts the anti-apoptotic activity of Bcl-2 causing cell death. On the other hand, if Bcl-2 is in excess, the Bcl2-Bax heterodimers or Bcl2-Bcl2 homodimers will predominate preventing cell death (Oltvai et al., 1993). Thus, the fate of the cell depends on the relative ratio and competitive dimerization between pro- and the anti-apoptotic proteins. The death-preventing effects of Bcl-2 and the death-promoting effects of Bax are dependent on their ability to insert and target the membranes. Removal of the membrane-anchoring domain (carboxy-terminal transmembrane region) reduces the efficacy of Bcl-2 and Bax as death regulators of apoptosis (Hockenbery et al., 1993; Zamzami et al., 1997)

Bcl-2 is the most potent physiological inhibitor of apoptosis and it has been shown to prevent cytochrome c release from the mitochondria (Lee et al., 2001; Sun et al., 2001), regulate calcium homeostasis (Lam et al., 1994; Marin et al., 1996), and act as an antioxidant (Hockenbery et al., 1993; Merad-Saidoune et al., 1999). Bcl-2, Bcl-xL and Bcl-w can inhibit apoptosis in response to many cytotoxic insults. Regardless of apoptotic stimuli, Bcl-2 is an integral membrane protein located in the outer mitochondrial membrane, the endoplasmic reticulum and the nuclear membrane (Gross et al., 1999).

The mechanism by which Bcl-2 proteins control mitochondrial membrane integrity are unclear. Two major models have been proposed: the permeability transition pore theory and the ion flow model (Gross et al., 1999). The permeability transition pore is a large pore permeable to molecules ~ 1500 daltons in size that regulates the potential of the inner mitochondrial membrane. Opening of the pore causes mitochondrial depolarization, uncoupling of oxidative phosphorylation and swelling of the mitochondria (Gross et al., 1999). The Bcl-2 family has been proposed to regulate the opening of the permeability transition pore. Pharmacological inhibitors of the permeability transition pore (cyclosporin A) inhibited Bax mediated cell death (Juergensmeier et al., 1998; Pastorino et al., 1998). The ion channel model that proposes that Bcl-2 proteins have ion channel activity was based on findings that three-dimensional structure of Bcl-xL, resembles the structure of bacterial toxins such as diphtheria that can insert into lipid bilayers and form channels capable of conducting ions (Muchmore et al., 1996). Moreover, Bcl-xL was shown to form cation-channels in lipid bilayers while Bax formed anion-channels (Budihardjo et al., 1999). Accordingly, the relative ration of pro or anti-apototic proteins could control the flow of ions.

The BH-3 domain is a critical death domain and is considered as the minimal domain required for pro-apoptotic proteins. There are a subset of pro-apoptotic proteins called the BH-3 only proteins that include Bad, Bid, Bik, Bim, Blk, Hrk, Blk, Noxa and Puma and which share sequence homology only within the BH-3 domain (Huang and Strasser, 2000). Another group of pro-apoptotic proteins, the Bax subfamily, share sequence homology to Bcl-2 in the BH1, BH2 and BH3 regions. Both subfamilies of proapoptotic proteins are required to initiate apoptosis. The BH3-only proteins act to detect death signs or intracellular damage and also function as antagonists of anti-apoptotic proteins. In the absence of an apoptotic stimulus, the BH3-only proteins are restrained by the cell in different ways. For example, Bim is bound to the microtubule motor protein dynein (Puthalakath et al., 1999), Bmf is bound to actin filaments (Puthalakath et al., 2001), phosphorylated Bad is sequestered by 14-3-3 scaffold proteins (Zha et al., 1996) and Bid is inactive until proteolytic cleavage (Li et al., 1998). After a death stimulus, these proteins are released from restraint and cause cell death. Following a death signal, Bim can dissociate from microtubule dynen, translocate to the mitochondria and antagonize antiapoptotic proteins such as Bcl-2 by inserting its BH3 domain into a groove on the antiapoptotic proteins (Puthalakath et al., 1999). Thus, the BH3-only proteins, neutralize their pro-survival counterparts. Bad is regulated by phosphorylation and dephosphorylation following a death signal and Bid becomes active after cleavage. Active caspase-8 cleaves Bid to release 15 and 11 kDa fragments (Li et al., 1998). The 15 kDa fragment of Bid translocates to the mitochondria and releases cytochrome c (Li et al., 1998; Madesh et al., 2002). Therefore, cleavage of Bid amplifies the apoptotic cascade induced by activation of death receptors.

Cysteine-Dependent Aspartate-Specific Proteinases (Caspases)

The cloning of ced-3 in *C. elegans* led to the discovery that this gene is homologous to the mammalian proteinase interleukin-1β converting enzyme, also referred as to caspase-1, responsible for the processing of the immature pro-interleukin-1β to its proinflammatory and biologically-active form (Thornberry et al., 1992). Even though caspase-1 is not involved in cell death, it was the first identified member of this large family of proteinases that plays a major role in inflammatory and apoptotic responses (Yuan et al., 1993; Xu et al., 1996). Caspases are considered highly specific proteinases that cleave at peptide bonds following aspartic acid residues and which use a cysteine side chain to catalyze the cleavage of peptide bonds (Stennicke and Salvesen, 1998). Caspases are constitutively and ubiquitously expressed as inactive proenzymes or zymogens that contain four domains: an N-terminal prodomain, a large subunit (~17-21 kDa), a C-terminal small subunit (10-13 kDa) and a short linker region between the large and the small subunit (Earnshaw et al., 1999). Caspases are activated in a sequential manner so that activation of one caspase leads to activation of other caspases. This cascade starts with autocatalytic activation of initiator caspases and is amplified through cleavage of downstream executioner caspases (Salvesen and Dixit, 1999). Inactive proenzymes become activated by two proteolytic cleavages at specific aspartic acid residues. The first cleavage occurs between the large and small subunits and removes the inter-domain linker region and the second cleavage removes the N-terminal prodomain. As a result, there is formation of a heterodimer containing one large and one small subunit (Wolf and Green, 1999). Crystallography studies have demonstrated that the active enzyme is actually a tetramer formed by two of these heterodimers (Rotonda et al., 1996).

Besides the requirement for an Asp residue at the carboxy-terminal (P1 site) of the cleavage site, caspases can be distinguished based on their substrate specificity determined by four amino acid residues located on the amino-terminal side of the cleavage site. The P4 site is the most critical determinant of substrate specificity

(Talanian et al., 1997; Thornberry et al., 1997). Therefore, caspases can be divided into three groups: Group I (caspases-1, -4 and -5) preferentially cleave after WEXD motif; Group II (caspase-2, -3, and -7) preferentially cleave after DEXD substrate and group III (caspases-6, -8 and -10) cleave after (I/L/V)EXD (Thornberry and Lazebnik, 1998). The specific cleavage sites for caspases-11, -12, -13 and -14 are not clear. Caspases are also classified according to their biological functions as inflammatory caspases (caspase-1, -4, -5, -11 and -13), initiators of apoptosis (caspases-2, -8, -9 and 10) and executioners/effectors of apoptosis (caspase-3, -6, and -7) (Stennicke and Salvesen, 2000). Caspases can also be distinguished based on the length of N-terminal domain. Initiators and the inflammatory caspases contain long prodomains up to over 100 amino acids while the prodomains in executioners caspases are short and usually less than 20 amino acids (Earnshaw et al., 1999). There are distinct motifs embedded in the N-terminal prodomain known as death effector domain (DED domain) and the caspase recruitment domain (CARD domain) (Stennicke et al., 1998).

A family of intracellular proteins collectively called inhibitor of apoptosis protein (IAP) functions as endogenous inhibitors of caspases. These proteins were first discovered in baculoviruses as suppressors of cell death in response to viral infection (Crook et al., 1993). In humans, this family is composed of six members: NAIP, C-IAP1/HIAP-2, Ciap-2/HIAP-1, XIAP/hILP, survivin, and BRUCE. Overexpression of XIAP, c-IAP1, c-IAP2, NIAP or survivin can suppress apoptosis induced by a variety of stimuli. There is evidence that XIAP, c-IAP1 and c-IAP-2 can block apoptosis by inhibiting caspase-3, -7 and -9 (Deveraux and Reed, 1999).

Intracellular Pathways of Caspase Activation

There are two major pathways of caspase activation, consisting of the mitochondrial- and receptor-mediated pathways. The mitochondrial, or intrinsic pathway as it is also called, has been described to occur in response to cellular stresses such as heat shock, oxidative stress, inhibition of protein kinase . In this pathway, disruption of the outer mitochondrial membrane by an apoptotic stimulus causes release of apoptogenic molecules such as cytochrome c, apoptosis-inducing factor (AIF) and smac/diablo (Du et al., 2000; Patterson et al., 2000). Once in the cytososl, cytochrome c associates with an adaptor molecule Apaf-1 (Li et al., 1997). The binding of cytochrome c to Apaf-1 results in a 10-fold increase in the affinity of Apaf-1 for dATP/ATP (Jiang and Wang, 2000). The binding of the nucleotide to Apaf-1/cytochrome c complex triggers oligomerization of Apaf-1 and formation a larger multimeric complex (~700 kDa) termed the apoptosome. As a result of the formation of the apoptosome, Apaf-1 undergoes a conformational change so that the N-terminal CARD sequence of Apaf-1 becomes exposed to interact with the CARD sequence of procaspase-9. The resulting CARD-CARD interaction leads to procaspase-9 autoproteolysis and self activation. Mature caspase-9 cleaves procaspase-3 to form caspase-3, the main executioner caspase (Gottlieb, 2000).

Results from gene knockout experiments have shown the importance of each component of the mitochondrial pathway . Elimination of Apaf-1, caspase-9 or caspase-3 inhibits most neuronal apoptosis during normal development (Kuida et al., 1996; Honarpour et al., 2000; Kuida, 2000). In cells in which cytochrome c was knocked out, Apaf-1 remained as a monomer after apoptotic stimuli (Li et al., 2000b). In Apaf-1 or caspase-9 knockout cells, there was no caspase activation after apoptotic stimuli even though cytochrome c was released from the mitochondria (Kuida et al., 1996; Honarpour et al., 2000).

Apoptosis-inducing factor (AIF) is a flavoprotein that resides in the intermembrane space of the mitochondria. Induction of apoptosis causes the release of AIF into the cytoplasm and translocation to the nucleus where it causes chromatin condensation and DNA-fragmentation (Susin et al., 1999). These effects, which are independent of caspase activation, indicates that mitochondrial damage can result in apoptosis even in the absence of caspase activation. Another mitochondrial factor, smac/diablo (second mitochondrial activator of caspases/direct IAP binding protein with low pI), was recently identified. This protein binds and neutralizes the caspase-inhibitory action of the IAPs (Du et al., 2000). The ability of smac/diablo to counteract the inhibition of IAPs provides a powerful way for the mitochondria to ensure rapid execution of apoptosis.

The extrinsic pathway of caspase activation is mediated by integral plasma membrane proteins called death receptors (DRs) that become activated upon binding of ligand. Death receptors are characterized by a C-terminal intracellular death domain (DD). The DD is a protein-protein interaction motif found in several receptors such as tumor necrosis factor receptor-1 (TNFR-1), Fas/CD95, DR3, DR4 and DR5 (Schmitz et al., 2000). The DD act as a recruitment domain for adaptor molecules that also contain DD. Examples of these adaptor molecules are Fas-associated protein with DD (FADD) and TNF-R1 associated death domain (TRADD). Some of these adaptor molecules contain other protein interaction domains, such as DED (death effector domain) found in

FADD and CARD. Initiator procaspases also contain DED (procaspase-8) and CARD (procaspase-9). The best studied death receptor is Fas. Binding of Fas by FasL induces receptor trimerization and recruitment of adaptor molecule FADD. The N-terminal DED motif of FADD is able to recruit procaspase-8 causing oligomerization and autoactivation since caspases can cleave one another when brought to close proximity. Active caspase-8 cleaves procaspase-3 to generate caspase-3 (Nagata, 1997; Earnshaw et al., 1999).

Both the mitochondrial and receptor-mediated mechanisms of cell death lead to activation of executioner caspases (caspase-3, -6, and -7) that cleave several intracellular substrates such as cytoplasmic proteins (lamin, actin, gelsolin), nuclear proteins (lamin) and proteins involved in DNA repair (poly-ADP-ribose polymerase) (Earnshaw et al., 1999). Caspase-3 plays an essential role in activating the latent, cytosolic endonuclease CAD (caspase-activated DNAse). Caspase-activated DNAse is usually sequestered in the cytoplasm via its binding to ICAD (inhibitor of CAD) which suppresses CAD endonuclease activity. During induction of apoptosis, caspase-3 cleaves ICAD from the CAD-ICAD complex (Enari et al., 1998; Sakahira et al., 1998). The free catalytic CAD is active, translocates to the nucleus and cleaves the DNA generating oligonucleosomal fragments (Enari et al., 1998; Sakahira et al., 1998).

Ceramide is an important second messenger involved in both mitochondrial and receptor-mediated pathways of caspase activation. Stress stimuli such as heat shock (Verheij et al., 1996; Kondo et al., 2000), oxidative stress (Goldkorn et al., 1998), ionizing radiation (Haimovitz-Friedman et al., 1994), chemotherapeutic agents (Strum et al., 1994) and activated death receptors such as TNFR-1 and Fas (Kronke, 1999) increase intracellular ceramide. These signals activate one or more sphingomyelin-specific forms of phospholipase C, called sphingomyelinase, which hydrolyzes the phosphodiester bond of sphingomyelin to generate ceramide (Haimovitz-Friedman et al., 1997; Pena et al., 1997; Sawai and Hannun, 1999). There are several isoforms of sphingomyelinases distinguished by pH optima. Acid sphingomyelinase (pH optimum 4.5-5.0) has been localized in acidic compartments such as lyzosome and endosome while magnesium-dependent neutral sphingomyelinases are present in the plasma membrane. Both acid and neutral sphingomyelinases are rapidly activated by several exogenous stimuli and can increase ceramide levels within few seconds or minutes (Chatterjee, 1999; Gatt, 1999)

Sphingomyelin is a phospholipid preferentially concentrated in the plasma membrane of mammalian cells. Most of the plasma membrane sphingomyelin is located in the outer leaflet of the membrane bilayer while the sphingomyelinase enzymes are located in the inner leaflet of the membrane and within lysosomes/endosomes. This cellular localization limits ceramide production in healthy cells. However, exposure to an apoptotic stimuli causes the translocation of sphingomyelin from the outer to the inner leaflet of the plasma membrane bilayer making sphingomyelin available for ceramide generation through actions of cytosolic neutral shingomyelinases (Tepper et al., 2000).

Ceramide can be deacetylated by ceramidases to generate sphingosine which is then phosphorylated by sphingosine kinase to form sphingosine-1-phosphate. Ceramide and sphingosine-1-phosphate act in opposing fashion. On the one hand, stress stimuli generate ceramide which activates the stress-activated protein kinases/c-jun N-terminal kinases (SAPK/JNK) leading to cell death (Verheij et al., 1996; Mosser et al., 1997; Buzzard et al., 1998). In contrast, growth factors stimulate ceramidases and sphingosine kinases to generate sphingosine-1-phosphate which activates the extracellular signal-regulated kinase (ERK) cascade leading to cell proliferation (Pena et al., 1997). In this way, the balance between intracellular levels of ceramide and sphingosine-1-phosphate is an important factor that determines whether the cell lives or dies.

Heat-Induced Apoptosis

Cellular insult activates two interconnected and yet opposing pathways: for cellular survival through induction of heat shock proteins and for apoptosis through activation of caspases. The balance of these two pathways modulates the cellular decision to live or die. Heat shock induces apoptosis in many cell types (Boreham et al., 1997; Gabai et al., 1997; Matsumoto et al., 1997; Mosser et al., 1997; Buzzard et al., 1998; McMillan et al., 1998; Gabai et al., 2000). For example, exposure of mouse embryo fibroblasts to 44°C for 10 min increased the percentage of apoptosis as compared to 37°C (Buzzard et al., 1998). In another study, heat shock of 40, 42 or 44°C for 30 min caused a linear increased in the percentage of HL-60 cells undergoing apoptosis as temperature increased (Kondo et al., 2000).

The mechanisms of heat-induced apoptosis are mediated by the second messenger ceramide in conjunction with the mitochondrial pathway of caspase activation. There is evidence that heat shock induces activation of neutral sphingomyelinases to generate ceramide through hydrolysis of sphingomyelin and induces cytochrome c release from the mitochondria (Mirkes and Little, 2000). Exposure of postimplantation mouse embryos to 45°C for 15 min induced release of cytochrome c from the mitochondria and induced caspase activation (Mirkes and Little, 2000). Heat shock of 40, 42 or 44°C for 30 min increased neutral magnesium-dependent sphingomyelinase activity, decreased sphingomyelin concentration and increased ceramide concentrations in HL-60 cells

(Kondo et al., 2000). Similarly, exposure of U937 cells to heat shock of 45°C increased ceramide levels and caused an parallel decrease in sphingomyelin (Verheij et al., 1996). Once ceramide level are increased, it can induce apoptosis by acting at different points of the apoptotic cascade. Ceramide induces Bax translocation to the mitochondria (Kim et al., 2001), reduces amount of Bcl-2 protein (Kim et al., 2001), reduces mitochondrial membrane potential (Ghafourifar et al., 1999), induces release of cytochrome c from the mitochondria (Ghafourifar et al., 1999; Kim et al., 2001), activates SAPK/JNK cascade (Verheij et al., 1996; Mosser et al., 1997; Buzzard et al., 1998) and increases expression of c-jun mRNA (Kondo et al., 2000). The role of Bax in mediating ceramide-induced apoptosis was demonstrated using Bax antisense mRNA. Treating HL-60 cells with Bax antisense mRNA reduced the ceramide-induced increase in the percentage of apoptotic cells, DNA-fragmentation and release of cytochrome c from the mitochondria (Kim et al., 2001). In addition, incubation of HL-60 cells with a broad spectrum caspase inhibitor prevented ceramide-induced apoptosis but did not affect Bax translocation to the mitochondria or cytochrome c release (Kim et al., 2001). Activation of SAPK/JNK has been implicated in heat-induced apoptosis mediated by ceramide. Exposure of mouse fibroblasts to 44°C for 10 min increased SAPK/JNK activation and this increase was blocked in thermotolerant cells (Buzzard et al., 1998). Moreover, both heat shock of 40°C or exogenous ceramide increased SAPK/JNK activity in the PEER human lymphoid tumor cell line (Mosser et al., 1997). Stress-activated protein kinases targets the transcription factors c-Jun, ATF2 and Elk1, and their phosphorylation and activation are associated with cell cycle delay and apoptosis (Chen et al., 1996; Verheij et al., 1996). The involvement of SAPK/JNK in stress-induced apoptosis was determined using a

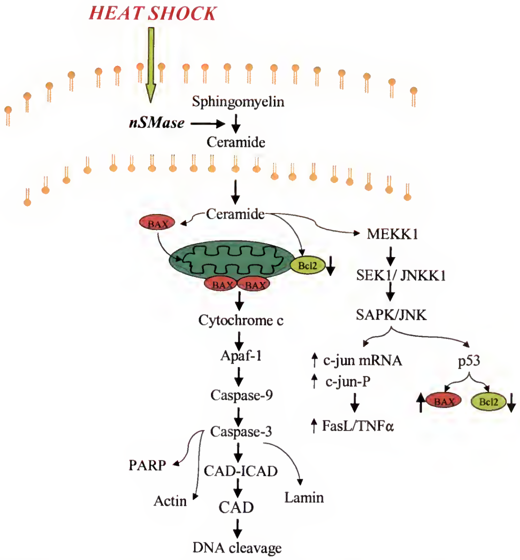

Figure 2-1. Heat-induced apoptosis. Heat shock induces activation of neutral sphingomyelinases to generate ceramide through hydrolysis of sphingomyelin. Ceramide causes a reduction in anti-apoptotic Bcl-2 protein in the mitochondria and induces translocation of pro-apoptotic Bax from the cytosol to the mitochondria. Once in the mitochondria Bax homodimers are formed to increase mitochondrial membrane permeability and allow the release of cytochrome c. Cytochrome c interacts with Apaf-1 to induce autoactivation of procaspase-9. Active caspase-9 cleaves procaspase-3 to generate caspase-3. Caspase-3 can cleave several substrates including actin, lamin, the DNA repair protein PARP and CAD-ICAD complex to generate active CAD which cleaves DNA. Ceramide also activates SAPK/JNK which regulates the transcription factor c-jun to induce FasL and TNF-α expression and activates p53 which up regulates Bax and down regulates Bcl-2 transcription.

nonphosphorylatable dominant-negative c-jun mutant lacking the N–terminal domain. Cells containing the c-jun mutation had increased ceramide levels and activation of SAPK/JNK activity after heat shock but were unable to undergo heat-induced apoptosis (Verheij et al., 1996). Moreover, c-jun antisense mRNA blocked heat- and ceramide-induced apoptosis in HL-60 cells (Kondo et al., 2000), indicating the involvement of this transcription factor mediating induction of apoptosis by heat. Phosphorylated c-Jun targets Fas ligand and tumor necrosis factor-α (Lee et al., 1995; Faris et al., 1998; Kolbus et al., 2000) promoters to induce expression of theses pro-apoptotic molecules. Moreover, SAPK/JNK can phosphorylate and activate the transcription factor p53 which translocates to the nucleus to up regulates Bax gene and down regulates Bcl-2 gene (May and May, 1999).

Given that heat shock increases generation of reactive oxygen species (ROS), it is likely that these molecules are implicated in induction of apoptosis. Indeed, ROS such as hydrogen peroxide can induce apoptosis in different cell types (Goldkorn et al., 1998; Witenberg et al., 1999). Cells treated with 150 μM hydrogen peroxide for 24 h had increased apoptosis while cells treated with 700 μM hydrogen peroxide for 4 h become necrotic (Guenal et al., 1997). The mechanisms involved in ROS-induced apoptosis are similar to heat-induced apoptosis. Hydrogen peroxide activated neutral- magnesium-dependent sphingomyelinase enzyme and induced rapid sphingomyelin hydrolysis to ceramide (Goldkorn et al., 1998). In addition, exogenous ceramide induced apoptosis with the same characteristics and the same time frame as hydrogen peroxide (Goldkorn et al., 1998). In another study, exposure of HepG2 cells to superoxide caused a rapid and massive release of cytochrome c from the mitochondria and this was accompanied the opening of the permeability transition pore. In contrast, blocking the voltage-dependent anion channel prevented superoxide-induced release in cytochrome c (Madesh and Hajnoczky, 2001).

Heat shock proteins have been implicated as major regulators of apoptosis responses induced by heat shock. Abrogation of HSP70 synthesis by antisense mRNA increased the proportion of Molt-4 tumor cells undergoing apoptosis (Wei et al., 1995). Target disruption of DNA binding activity of the transcription factor for heat shock proteins (HSF-1) prevented heat-induced expression of inducible HSP70. Moreover, induction of thermotolerance reduced heat-induced TUNEL staining in wild type cells but not in cells containing the HSF-1 mutation (McMillan et al., 1998). This experiment demonstrates that HSPs are implicated in protection against heat-induced apoptosis. Heat shock proteins act at different points in the apoptotic cascade to exert a negative regulation. In one of the first studies to indicate a role for HSP70 in regulation of apoptosis, it was reported that PEER human lymphoid tumor cells expressing HSP70 had reduced SAPK/JNK activity after heat shock and were more resistant to heat-induced apoptosis as determined by reduced cleavage of the caspase substrate poly (ADP-ribose) polymerase (PARP) (Mosser et al., 1997). In other studies, induction of thermotolerance or exogenous expression of HSP72 protected PEER human lymphoid tumor cells or fibroblasts from heat-induced apoptosis and inhibited SAPK/JNK activation (Gabai et al., 1997; Buzzard et al., 1998). High concentrations of the constitutive and inducible forms of HSP70 protected cells from heat-induced apoptosis by preventing SAPK/JNK, procaspase-9 and procaspase-3 activation (Mosser et al., 2000).

Recent studies have focused on the role of HSPs on apoptosome formation. Experiments showed that HSP70 and HSP90 coprecipitated with Apaf-1 in heat-shocked cells but not in control cells (Saleh et al., 2000). Heat shock protein 70 can inhibit caspase processing by direct binding with Apaf-1 to prevent assembly of the apoptosome (Beere et al., 2000; Saleh et al., 2000). According to Salesh et al., (2000), HSP70 interacts with the CARD sequence of Apaf-1 to inhibit oligomerization of Apaf-1 and subsequent recruitment of procaspase-9. Overexpression of HSP70 prevented serum-withdrawl induced apoptosis in Apaf-1$^{(-/-)}$ cells, indicating that Apaf-1 is not the only target for HSP70. There is also evidence that HSP70 interacts with AIF to prevent chromatin condensation induced by AIF and that HSP70 protected cells from AIF-induced apoptosis (Ravagnan et al., 2001). Additionally, antisense HSP70 complementary DNA (which reduced the expression of endogenous HSP70), made cells more sensitive to AIF (Ravagnan et al., 2001).

Constitutive expression of HSP27 increased resistance of cells to apoptosis induced by Fas and staurosporine (Mehlen et al., 1996). The HSP27 differentially regulates the apoptosome. Heat shock protein 27 binds cytochrome c released from the mitochondria and prevents binding of cytochrome c to Apaf-1 and subsequent procaspase-9 activation after heat shock (Bruey et al., 2000).

Apoptosis in Preimplantation Embryonic Development

Light and electron microscopy studies have shown the presence of dead or dying cells in the blastocysts of mice (El-Shershaby and Hinchliffe, 1974; Copp, 1978; Handyside and Hunter III, 1986), humans (Hardy et al., 1989; Hardy, 1999), cows (Mohr and Trouson, 1982; Plante and King, 1994) and baboons (Enders et al., 1990). Indeed, a certain proportion of blastomeres undergo apoptosis as a normal feature of development. In species such as the mouse (El-Shershaby and Hinchliffe, 1974; Handyside and Hunter III, 1986), human (Hardy et al., 1989) and rat (Pampfer, 2000), spontaneous cell death does not seems to occur during the first four rounds of cell division, but instead is mostly observed during blastocyst formation. A time course experiment to determine the onset of spontaneous apoptosis in mouse embryos demonstrated that TUNEL-positive cells were not present in preimplantation mouse embryos at different stages of development up to the morula stage regardless of whether embryos were produced in vivo or in vitro embryos (Kamjoo et al., 2002). Rather, TUNEL staining was first observed at the blastocyst stage. In bovine embryos, spontaneous apoptosis, as determined by TUNEL staining, was first observed in embryos at the 9-16 cell stage coincident with the time of embryonic gene activation (Byrne et al., 1999; Matwee et al., 2000). There is also evidence from rodent (Brison and Schultz, 1997; Pampfer, 2000) and cattle (Byrne et al., 1999; Neuber et al., 2000) blastocysts that cells from the inner cell mass have increased rates of apoptosis as compared to trophectoderm cells. It is possible that apoptosis is a strategy to regulate the inner cell mass to remove cells that retain the potential to form trophectoderm.

Even though spontaneous apoptosis is a developmentally-regulated phenomenon, there is evidence that the apoptotic machinery is constitutively expressed in oocytes and early embryos. TUNEL staining associated with metaphase II chromosomes or chromatin was detected in 2% (6/351) of mouse oocytes and 8% (8/215) of human oocytes (van Blerkom and Davis, 1998). In addition, culture of mouse embryos at 1-4 cell stage (Well et al., 1996) or bovine embryos at 1-16 cell stage (Matwee et al., 2000) in the presence of the protein kinase inhibitor staurosporine induced apoptosis in these early stages as detected by TUNEL staining. In another study, treatment of mouse zygotes with staurosporine induced activity of group II caspases (caspase-2, -3 and -7) (Exley et al., 1999). Apoptosis could also be induced in mouse zygotes exposed to exogenous hydrogen peroxide (Liu et al., 2000). However, certain aspects of cell death are altered in early embryos since the time required for staurosporine to induce apoptosis was longer for 1-4 cell stage embryos (26-60 h) than for embryos at the blastocyst stage (18 h).

Like in other cell types, embryonic apoptosis is regulated by caspases and anti- and pro-apoptotic molecules. Reverse transcriptase polymerase chain reaction revealed that mRNA for initiator caspases-2 and effector caspase-3 and -6 were expressed in mouse oocytes and embryos from the 2-cell to the blastocyst stage. In contrast, mRNA for inflammatory caspases-1 and -11 were expressed only in oocytes (Exley et al., 1999). In addition, CAD and caspase-3 mRNA and proteins were detected in rat blastocysts (Hinck et al., 2001). Similarly, transcripts for the anti-apoptotic Bcl-2, Bcl-xL and Bcl-w and the proapoptotic Bax and Bak were expressed in oocytes and embryos from zygote to the blastocyst stage (Exley et al., 1999). The abundance of mRNA for Bcl-x and Bcl-2 was higher in mouse embryos at the 1-cell stage and decreased as the embryo advanced in development (Juriscova et al., 1998). Experiments using α-amanitin indicated that most of the Bcl-x and Bcl-2 mouse transcripts detected at the 1-cell stage were from maternal origin (Juriscova et al., 1998). In contrast to the expression pattern of anti-apoptotic proteins, transcripts for Bax and Bad were low in zygotes and two-cell embryos and increased through the blastocyst stage (Juriscova et al., 1998). Thus, the ratio between anti- and pro-apoptotic molecules may be involved with the lack of apoptosis in early cleavage stage embryos. Western blotting analysis indicated that the amount of Bcl-2 protein was higher than Bax protein in good quality bovine embryos at the 2-8 cells and blastocyst stages whereas the amount of Bax protein was higher in fragmented embryos (Yang and Rajamahendran, 2002).

Phosphorylation and activation of the tumor suppressor gene p53 results in translocation of this transcription factor to the nucleus where it up regulates Bax gene and down regulates Bcl-2 gene (May and May, 1999). Both p53 mRNA and protein have been detected in bovine embryos throughout the preimplantation period. However, the p53 protein was restricted to the cytoplasm even in TUNEL-positive blastomeres (Matwee et al., 2000). This observation indicates that spontaneous apoptosis in bovine embryos may be independent of transcriptional activity of p53.

Even though cell death by apoptosis is a conserved process observed in embryos produced in vivo and in vitro, there is evidence that embryos produced in vitro have a higher proportion of apoptotic cell death than embryos produced in vivo. For example, 0.7% of blastomeres from MF1 mouse embryos flushed from the oviduct tract were apoptotic while 8.7% of blastomeres of cultured embryos were apoptotic (Kamjoo et al., 2002). It is likely that the culture environment modulates cell death. Oxidative stress can trigger embryonic apoptosis since culture of bovine embryos under low oxygen tension (5% O_2) increased the rate of blastocyst formation and decreased the proportion of apoptotic cells in blastocysts as compared to embryos cultured under high oxygen tension (20% O_2) (Van Soom et al., 2002). Exposure of mouse zygotes to H_2O_2 induced apoptosis characterized by cell shrinkage, diffuse cytochrome c distribution in the cytoplasm consistent with the release of cytochrome c from the mitochondria, positive staining for active caspase-3, decrease in mitochondrial membrane potential and TUNEL staining in condensed pronuclei (Liu et al., 2000). In human embryos, the concentration of H_2O_2 detected was higher in fragmented embryos than in non-fragmented embryos (Yang et al., 1998).

The role of the cytoplasm in oxidative-induced apoptosis was tested using reconstructed mouse zygotes. Zygotes were reconstructed using either H_2O_2-treated cytoplasm or H_2O_2-treated pronuclei. Results indicated that the cytoplasm of zygotes treated with H_2O_2 could transfer the apoptotic signal to zygotes with untreated pronuclei while untreated cytoplasm could rescue pronuclei from oxidative stress (Liu and Keefe, 2000). Given the central role of mitochondria on free radical production (Thompson et al., 2000) and apoptosis (Gottlieb, 2000), it is likely that this organelle represents the cytoplasmic component regulating oxidative-stress induced apoptosis.

Like for other cell types, HSPs are likely to play a role in protecting embryos from cell death. Addition of HSP70 antibody to the culture medium of bovine embryos from day 3 to day 9 reduced development to the blastocyst stage and increased TUNEL-positive cells as compared to controls without antibody (Matwee et al., 2001). This observation suggests either the antibody crosses the plasma membrane or there are membrane receptors for HSP70. When bovine blastocysts were exposed to 42°C for 6 h there was a reduction in the proportion of TUNEL-positive cells from 7.6% in control embryos to 4.8% in the heat-shocked blastocysts (Matwee et al., 2001). Considering that blastocysts are very resistant to heat shock, it is possible that 42°C for 6 h induced sufficient HSP70 to inhibit apoptosis.

Growth and survival factors are key regulators of the cellular decision to live or die. Initial evidence for the role of growth factors in embryonic development came from coculture experiments. Culture of bovine embryos with somatic cells stimulated embryonic development (Kajihara et al., 1990; Kajihara et al., 1991; Thibodeaux et al., 1992). It has also been demonstrated that coculture of mouse embryos with human oviductal cells reduced the incidence of apoptosis in embryos at the morula and blastocyst stage (Xu et al., 2000). Likewise, culture of mouse embryos at high density stimulated development to the blastocyst stage and reduced percentage of apoptotic cells (Brison and Schultz, 1997; O'Neill, 1998).

There is also direct evidence for the role of growth factors in rescuing embryos from cell death. Addition of exogenous transforming growth factor-α to culture medium of single mouse embryos reduced the percentage of apoptotic cells without affecting development or total cell number (Brison and Schultz, 1997). In addition, transforming growth factor-α-deficient embryos had increased blastocyst apoptosis (Brison and Schultz, 1998). Taken together, these results indicated that transforming growth factor-α act as a survival factor minimizing the incidence of cell death in mouse embryos. Proteins of the insulin and insulin-like growth factors (IGF) family are also involved in regulation of embryo development and death. Addition of IGF-I to culture medium of bovine zygotes stimulated development to the blastocyst stage (Byrne et al., 2002; Makarevich and Markkula, 2002; Moreira et al., 2002), increased blastocyst cell number (Byrne et al., 2002; Makarevich and Markkula, 2002; Moreira et al., 2002) and decreased the proportion of blastomeres that were apoptotic (Byrne et al., 2002; Makarevich and Markkula, 2002). In contrast, IGF-II had no effect on apoptosis but increased blastocyst development and embryo cell number while insulin decreased apoptosis without affecting blastocyst cell number (Byrne et al., 2002). When growth hormone was added to culture medium of bovine embryos, there was increased development to the blastocyst stage (Moreira et al., 2002), increased blastocyst cell number (Kolle et al., 2002; Moreira et al., 2002), decreased blastocyst apoptosis (Kolle et al., 2002) and reduced immunoreactive Bax protein staining in bovine blastocysts (Kolle et al., 2002). In human embryos, IGF-I increased development to the blastocyst stage, decreased the proportion of apoptotic cells without affecting total blastocyst cell number (Spanos et al., 2000).

Reactive Oxygen Species

During aerobic energy production, synthesis of ATP is coupled to the reduction of oxygen to water by the addition of four electrons. Not all oxygen is completely reduced during metabolism, however. Approximately 1-2% of metabolized oxygen is converted to ROS (Kowaltowski and Vercesi, 1999; Cadenas and Davies, 2000). Incomplete reduced oxygen species, including superoxide anion ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) (Sies, 1985; Halliwell and Gutteridge, 1990) (see Figure 2-2), are very reactive molecules that act to strip electrons from other constituents of the cell and cause cellular damage. Reactive oxygen species can be produced by various enzymatic reactions in the cell. For example, $^{\bullet}O_2^-$ can be formed by the one-electron reduction of oxygen during electron transport in mitochondria and by oxidases such as xanthine oxidase in the cytoplasm (Kuppusamy and Zweier, 1989), NADPH-oxidase in phagocytic cells (Vignais, 2002), cyclooxygenase in the membranes and lipooxygenase in peroxisomes (Kontos et al., 1985; McIntyre et al., 1999). Even though $^{\bullet}O_2^-$ is only a mild oxidant, it is the most abundant radical produced in the cell. It can react with cellular

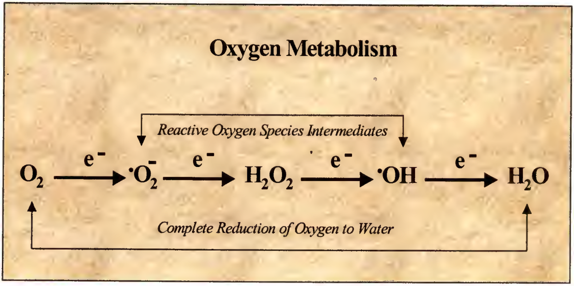
Oxygen Metabolism
Reactive Oxygen Species Intermediates
$O_2 \xrightarrow{e^-} {}^{\bullet}O_2^- \xrightarrow{e^-} H_2O_2 \xrightarrow{e^-} {}^{\bullet}OH \xrightarrow{e^-} H_2O$
Complete Reduction of Oxygen to Water

Figure 2-2. Oxygen metabolism. The reactive oxygen species superoxyde radical (${}^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical (${}^{\bullet}OH$) are formed from stepwise oxygen acceptance by one, two and three electrons, respectively

components such as lipids and thiol groups or be dismutated spontaneously or by the enzyme superoxide dismutase to generate H_2O_2. Hydrogen peroxide is also a mild oxidant that can directly oxidize biological molecules. In the presence of free transition metals, hydrogen peroxide and superoxide can produce through the Fenton reaction the most harmful and reactive metabolite, hydroxyl radical (Cuzzocrea et al., 2001). Hydroxyl radical is formed by the reaction of hydrogen peroxide with transition metals such as iron or copper ($H_2O_2 + Fe^{2+}/Cu^{+} \Rightarrow {}^{\bullet}OH + OH^{-} + Fe^{3+}/Cu^{2+}$). Then, superoxide plays a role in recycling the transition metals to allow the generation of more hydroxyl radicals ($Fe^{3+}/Cu^{2+} + {}^{\bullet}O_2{-} \Rightarrow Fe^{2+}/Cu^{+} + O_2$). The sum of both reactions is known as the Häber-Weiss reaction (Cuzzocrea et al., 2001).

Oxygen metabolities can damage several cellular components such as lipids, proteins and DNA One of the most damaging cellular effects of free radicals is lipid peroxidation because of the chain reaction of lipid consumption and amplification which generates more reactive products (Figure 2-3). Lipid peroxidation is initiated by a hydroxyl radical-mediated abstraction of a hydrogen atom from a membrane polyunsaturated fatty acid (LH) or free fatty acid to result in the formation of lipid radical ($L^{\bullet}$). Once the lipid radical is formed, it tends to stabilize by molecular rearrangement to form a conjugated diene. Under aerobic conditions, conjugated diene rapidly combine with oxygen to give a peroxyl radical ($LOO^{\bullet}$) that propagates the oxidative process (Dargel, 1992). The peroxyl radical is able to abstract H from another lipid molecule to cause an autocatalytic chain reaction of oxidative steps generating other free radical molecules (Allen, 1991), toxic alcohols, ketones, and aldehydes (Dargel, 1992). The peroxyl radical combines with H to give a lipid hydroperoxide (LOOH). Formation of lipid hydroperoxide results in changes in membrane fluidity and permeability. The oxidative chain reaction is also continued when lipid hydroperoxide interacts with iron-containing compounds to generate an alkoxyl radical (LO•). Termination occurs by the action of antioxidants such as vitamin E or by the recombination of radicals resulting in the formation of non-radical products such as aldehydes (malondialdehyde) and other fragmentation products (Comporti, 1989; Baskin and Salem, 1997).

The rate and extent of lipid oxidation depends on the lipid composition of the membrane. The polyunsaturated fatty acids are particularly susceptible to lipid peroxidation because removal of a hydrogen atom by a ROS is easier as the number of double bonds in a fatty acid chain increase (Halliwell and Chirico, 1993). On the other hand, cholesterol can act as an antioxidant by reducing the proximity between oxygen species and their targets to minimize the propagation of a chain reaction since cholesterol radical has reduced reaction rate (Sevanian and Ursini, 2000).

Reactive oxygen species can also modify amino acid residues, especially sulfur-containing residues, to cause protein sulphydryl oxidation, disulphide bond formation, protein aggregation or fragmentation and loss of biological activity (Dargel, 1992). Even though protein peroxidation processes do not proceed in a chain reaction, recent evidence indicates that protein radicals are not static end-products of protein oxidation. Instead, protein radicals are dynamic molecules that can further oxidize molecules in their surroundings. For instance, the bovine serum albumin radical oxidized linoleic acid in vitro to generate lipid hydroperoxides and conjugated dienes (Ostdal et al., 2002). From a quantitative point of view, free radical-mediated DNA damage is the least significant. However, its importance resides in the genetic consequences of such damage.

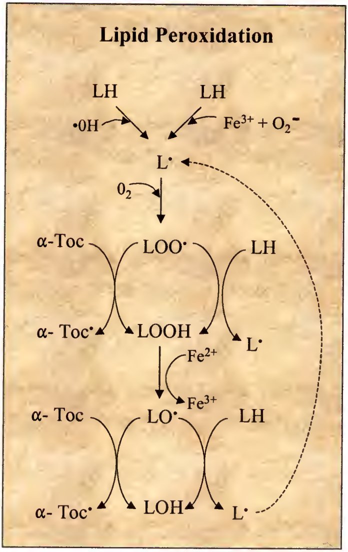

Figure 2-3. Lipid peroxidation. Abstraction of H atom from a polyunsaturated fatty acid (LH) can be directly triggered by hydroxyl radical or can be mediated by superoxide anion in the presence of a transition metal. This step generates a lipid radical ($L^\bullet$) which tends to stabilize by molecular rearrangement to form a conjugated diene. Under aerobic condition, conjugated diene combine with oxygen to give a peroxyl radical ($LOO^\bullet$). The peroxyl radical combines with an H either donated from a chain breaking α-tocopherol or from a polyunsaturated fatty acid to give a lipid hydroperoxide (LOOH). Lipid hydroperoxide can interact with iron-containing compounds to generate an alkoxyl radical ($LO^\bullet$) that continues the oxidative chain reaction. Termination occurs by the action of α-tocopherol or by the recombination of radicals resulting in the formation of non-radical products.

Endogenous DNA damage is genotoxic and induces mutations that can lead to disorders such as cancer, atherosclerosis and acquired immunodeficiency syndrome (Olinski et al., 2002). Reactive oxygen species attacks either the purine and pyrimidines bases or deoxyribosyl DNA backbone to cause DNA strand breaks, DNA-protein cross links, oxidation of purines, base-pair mutations, rearrangements, insertions and deletions (Cuzzocrea et al., 2001). The mitochondrial DNA is susceptible specially to oxidative damage. The continuous generation of ROS by the mitochondrial respiratory chain makes the mitochondria the major source of ROS production and accumulation (Turrens, 1997). Moreover, the lack of histone proteins to protect the mitochondrial DNA against ROS, lack of proofreading mechanism, and an inefficient DNA repair mechanism makes the mitochondria an easy target for damage by ROS (Kowaltowski and Vercesi, 1999).

In addition, there is evidence that ROS causes apoptosis (Skulachev, 1998; Yang et al., 1998; Abramova et al., 2000). Induction of apoptosis by ROS may occur as a consequence of ROS-mediated DNA damage (Abramova et al., 2000) as well as ROS-mediated increased membrane permeability and leakage of cytochrome c (Skulachev, 1998).

Antioxidants

The balance between the production and disposal of oxidant molecules is essential for tissue homeostasis. Cells have a variety of antioxidant systems to maintain ROS in check. The first line of antioxidant defense are enzymatic systems to prevent accumulation of ROS and initiation of oxidative stress. Included in this line of defense are enzymes that convert ROS to water. Superoxide dismutase converts superoxyde to hydrogen peroxide. In mammals, two form of superoxide dismutase have been identified:

manganese superoxide dismutase which is located in the mitochondria, and copper-zinc superoxide dismutase, which is mostly located in the cytoplasm (Harris, 1992). Hydrogen peroxide is converted enzymatically to water by either glutathione peroxidase in cytoplasm or catalase in peroxisomes. Glutathione peroxidase is a selenium dependent-enzyme that contains 4-atoms of selenium per molecule and utilizes reduced GSH to convert hydrogen peroxides to water or lipid peroxides to alcohols (Arthur, 2000). Selenoperoxidases contain selenocysteine at their active site, and this amino acid is involved in the catalytic cycle since selenium is oxidized by a hydroperoxide to a selenenic acid derivative (Zachara, 1992). Five glutathione peroxidases subtypes have been identified including the cytosolic glutathione peroxidase (GPX-1), a cytosolic gastrointestinal glutathione peroxidase (GPXG1) which is closely related to GPX-1, a glycoprotein form of glutathione peroxidase (GPX-3) in plasma, a phospholipid hydroperoxide glutathione peroxidase (GPX-4) (Arthur, 2000) and the sperm nucleus glutathione peroxidase (snGPX) (Pfeifer et al., 2001). Glutathione peroxidase (GPX-1) knockout mice were healthy animals with normal phenotype (Ho et al., 1997) but were more susceptible to ceratin forms of stress (Yoshida et al., 1997). In addition to enzymatic antioxidants, metal-binding macromolecules such as transferrin and albumin are present in extracellular fluid and act as metal chelators to remove free metal catalysts of ROS reactions (Halliwell, 1987).

A second line of antioxidant defense involves scavenger molecules that neutralize free radical activity by serving as electrons donors. These molecules include chain breaking lipid-soluble antioxidants such as vitamin E (Kaikkonen et al., 2001; O'Leary et al., 2001; Suzumura et al., 2001), coenzyme Q (Thomas et al., 1996; Nohl et al., 1999) and β-carotene (Zhang and Oaye, 2000) as well as water-soluble antioxidants such as ascorbic acid (Lykkesfeldt, 2002; Marathe and Mishra, 2002), GSH (Meister, 1983; Lykkesfeldt, 2002) and hypotaurine (Shi et al., 1997) in the cytoplasm and α-lipoic acid which is soluble in both lipid and aqueous environments (Gonzalez-Perez et al., 2002).

Vitamin E is the major antioxidant in membranes with greatest concentrations found in the membranes of the Golgi apparatus and lysosomes (Wang and Quinn, 2000). This generic term vitamin E refers to a group of chain-breaking antioxidants including four tocopherols (α, β, γ and δ) and four tocotrienols (α, β, γ and δ) (IUPAC-IUB, 1981). Among these, α-tocopherol is the most abundant form and has the highest biological activity (Wang and Quinn, 2000). Like other members of the vitamin E family, α-tocopherol protects polyunsaturated fatty acids in membranes from lipid peroxidation (Kaikkonen et al., 2001; Gonzalez-Perez et al., 2002) through donation of electrons to lipid peroxyl radical to form a lipid hydroperoxide and the α-tocopherol radical. Oxidized α-tocopherol can be restored to the active reduced form by other antioxidants (Figure 2-4). For example, vitamin E can be directly regenerated by ascorbic acid (Guo and Packer, 2000), GSH (Kagan and Tyurina, 1998) and ubiquinol (Lass and Sohal, 1998) and indirectly regenerated by dihydrolipoic acid (Packer, 1998; Guo and Packer, 2000) (Figure 2-4). Failure to reduce oxidized α-tocopherol can lead to accumulation of α-tocopherol-radical that acts as a prooxidant (Kontush et al., 1996).

Production of Reactive Oxygen Species in Preimplantation Embryos

Reactive oxygen species such as superoxide anion and hydrogen peroxide have been detected in mouse (Nasr-Esfahani et al., 1990; Goto et al., 1993; Joo et al., 2001) and rabbit (Manes and Lai, 1995) preimplantation embryos. One source of free radicals is

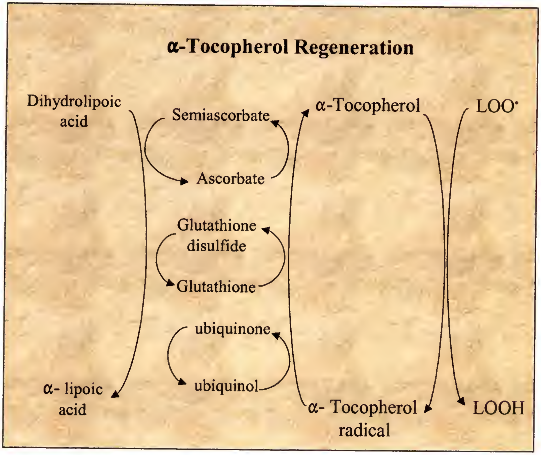

Figure 2-4. Regeneration of α-tocopherol from α-tocopherol radical. The α-tocopherol radical can be reduced to α-tocopherol through interaction with other antioxidants including ascorbate, glutathione and ubiquinol. Any these antioxidants can directly regenerate α-tocopherol radical. Moreover, dihydrolipoic acid can indirectly regenerate α-tocopherol radical through recycling of semiascorbate, glutathione disulfide and ubiquinone to their reduced form.

likely to be leakage of electrons during electron transfer reactions in the mitochondria. Like most cells, embryos produce energy by generating ATP via the conversion of glucose to lactate by glycolysis and the oxidation of substrates via the TCA cycle and oxidative phosphorylation (Thompson et al., 2000). Transient inhibition of mitochondrial ATP production using inhibitors or uncouplers of oxidative phosphorylation increased development in bovine (Thompson et al., 2000) and porcine (Machaty et al., 2001) embryos. Such an effect could be mediated possibly by reduction in ROS leakage during oxidative phosphorylation. Oxidases such as xanthine oxidase and NADPH oxidase are also involved in generation of ROS in embryos. Addition of xanthine oxidase or hypoxanthine to culture medium of bovine embryos reduced development to the blastocyst stage and blastocyst cell number (Iwata et al., 1999). Inhibition of xanthine oxidase decreased ROS in mouse embryos (Nasr-Esfahani and Johnson, 1991) and increased development of bovine embryos to the blastocyst stage (Iwata et al., 1999). Similarly, inhibition of NADPH oxidase in mouse 2-cell embryos caused a reduction in hydrogen peroxide (Nasr-Esfahani and Johnson, 1991) whereas addition of NADPH oxidase generated superoxide and hydrogen peroxide in rabbit blastocysts (Manes and Lai, 1995).

Environmental factors such as oxygen tension (Goto et al., 1993; Nagao et al., 1994; Nakayama et al., 1994), visible light (Goto et al., 1993; Nakayama et al., 1994) and transition metals (iron and cooper) (Nasr-Esfahani et al., 1999) enhance production of ROS by cultured embryos. For example, culture of bovine (Nagao et al., 1994) and mouse (Goto et al., 1993) embryos under atmospheric oxygen concentration (20% O_2) increased generation of ROS as compared to embryos cultured in 5% O_2. Moreover, embryos cultured under 5% O_2 tension had higher development than embryos cultured in atmospheric oxygen for both bovine (Fujitani et al., 1997; Lim et al., 1999) and mouse (Goto et al., 1993) embryos.

The aforementioned experiments imply that ROS can damage preimplantation embryos. Other evidence that this is the case include one experiment showing that addition of ROS compromises embryo development. Addition of hydrogen peroxide to the culture medium of mouse zygotes caused developmental arrest and cell death by apoptosis characterized by DNA strand breaks and mitochondrial alterations such as depolarization, reduced membrane potential and cytochrome c release (Liu and Keefe, 2000; Liu et al., 2000). In fragmented human embryos, increased hydrogen peroxide concentrations were associated with apoptosis (Yang et al., 1998). Exposure of cultured bovine zygotes to hydrogen peroxide reduced development to the blastocyst stage in a dose-dependent manner (Morales et al., 1999). Moreover, addition of a free radical generator 2,2' -azobis (2- amindinopropane) dihydrochloride to culture medium decreased development to the blastocyst stage in embryos cultured under 5% or 20% oxygen environment (Fujitani et al., 1997).

There is also evidence that antioxidants can minimize the deleterious effects of ROS on embryonic development. Addition of GSH to the culture medium of in vitro produced mouse (Legge and Sellens, 1991; Gardiner and Reed, 1994) bovine (Luvoni et al., 1996) and goat (Lee et al., 2000) embryos stimulated development. Addition of cysteamine, an inducer of glutathione synthesis, during in vitro maturation of ovine oocytes increased the proportion of embryos that developed to the morula and blastocyst stage (de Matos et al., 2002). Similarly, addition of vitamin E to culture medium stimulated development of bovine zygotes to the expanded blastocyst stage under 5% oxygen culture environment (Olson and Seidel Jr, 2000). In contrast, addition of histidine (Legge and Sellens, 1991), taurine (Lonergan et al., 1999), vitamin C (Olson and Seidel Jr, 2000) and ethylenediaminetetracetic acid (EDTA) (Olson and Seidel Jr, 2000) to culture media of mouse and bovine embryos had no beneficial effect on development. Moreover, addition of enzymatic antioxidants to culture medium can also have a beneficial effect on development to the blastocyst stage. This has been shown in mouse embryos for superoxide dismutase (Payne et al., 1992; Chun et al., 1994; Orsi and Leese, 2001) and catalase (Orsi and Leese, 2001. The beneficial effects of superoxide dismutase varied according to mice strain (Payne et al., 1992), suggesting genetic variation in ROS production.

Antioxidant Systems in Preimplantation Embryos

Like for other cells, glutathione is a major cytosolic antioxidant in embryos. In the mouse embryo intracellular glutathione content is high in oocytes, decreases after fertilization and continues to decrease as embryos develop to the blastocyst stage. (Gardiner and Reed, 1994). Moreover, transcripts for glutamylcysteine synthetase, the rate-limiting enzyme in glutathione biosynthesis, were present in mouse embryos from zygote to blastocyst stage (Harvey et al., 1995). Indeed, mouse embryos can synthesize glutathione as early as the 2-cell stage (Gardiner and Reed, 1995). In contrast, in bovine embryos, intracellular glutathione concentrations decreased from the zygote to the 2-8 cell stage and increased at the 9-16 cell stage reaching the highest concentrations at the hatched-blastocyst stage (Lim et al., 1996). Transcripts for the bovine glutamylcysteine were detected from zygote to the morula stage but were not detected at the blastocyst stage (Harvey et al., 1995). Glutathione content was lower in mouse embryos produced in vitro as compared to their in vivo counterparts (Gardiner and Reed, 1994), suggesting that in vitro produced embryos undergo oxidative stress.

Expression of genes encoding antioxidant enzymes has been shown in mouse (Harvey et al., 1995) and bovine embryos (Harvey et al., 1995; Lequarré- et al., 2001). Transcripts for catalase, cooper-zinc-superoxide dismutase, manganese-superoxide dismutase, glutathione peroxidase and glutamylcysteine synthetase were identified by RT-PCR in mouse embryos produced in vivo and in vitro from the zygote to blastocyst stage (Harvey et al., 1995). Similarly, transcripts for catalase, cooper-zinc-superoxide dismutase and glutathione peroxidase (Harvey et al., 1995)were detected in in vitro-produced bovine embryos from the zygote to the blastocyst stage whereas manganese-superoxide dismutase was only expressed in bovine zygotes and embryos at the 5- to 8-cell stage (Lequarré- et al., 2001). For bovine embryos produced in vivo, cooper-zinc-and manganese-superoxide dismutase mRNAs were detected at the morula and blastocyst stage (Lequarré- et al., 2001). In addition, proteins for cooper-zinc-and manganese-superoxide dismutase were immunodetected in bovine zygotes and blastocysts (Lequarré- et al., 2001).

The Role of Reactive Oxygen Species and Antioxidants During Heat Shock

Some of the detrimental effects of elevated temperature on cellular function have been associated with increased free radical production. Heat shock can increase the metabolic rate of cells resulting in increased oxygen consumption and enzymatic activity. Oxygen consumption was stimulated in rat liver following exposure to 42°C as compared to 37°C (Skibba and Collins, 1978; Skibba et al., 1986) and heat shock stimulated activity of free-radical producing enzymes such as xanthine oxidase in liver (Skibba and Stadnicka, 1986; Skibba et al., 1989b) and cyclooxygenase in endometrium (Malayer et al., 1990). A study using electron paramagnetic resonance spin trapping to measure ROS showed that exposure of rat intestinal epithelial cells to heat shock increased the flux of cellular free radicals (Flanagan et al., 1998). Similarly, heat shock of perfused liver increased release of hydroperoxides (Skibba and Stadnicka, 1986; Skibba et al., 1989a). Exposure of CHO cells to heat shock of 42°C, decreased amounts of reduced GSH and increased amounts of oxidized GSSG, suggesting increased free radical generation (Lord-Fontaine and Averill-Bates, 2002). Moreover, GSH depletion made cells more sensitive to elevated temperature (Mitchel et al., 1983; Russo et al., 1984). In addition, intracellular GSH concentration was decreased in mouse morulae following heat shock (Aréchiga et al., 1995), suggesting heat shock stimulated utilization of GSH reducing potential.

There is some evidence that antioxidants exert a protective effect on heat-shocked embryos. Stimulation of GSH synthesis by adding S-adenosyl-methionine to culture medium of mouse morulae decreased the deleterious effects of 43°C on embryonic viability and development to the blastocyst stage (Aréchiga et al., 1995). A similar beneficial effect on development was observed when taurine was added to the culture medium of mouse embryos exposed to 42°C (Malayer et al., 1992). In contrast, no beneficial effects of glutathione or vitamin E were observed on development. In one study, addition of vitamin E or GSH to culture medium of mouse morula exposed to 43°C increased embryonic viability but had no effect on subsequent development (Aréchiga et al., 1994a). Similarly, addition of GSH, taurine or a membrane-permeable GSH ester to culture medium of bovine 2-cell embryos did not alleviate the negative effects of heat shock on embryonic development (Ealy et al., 1995). Thus, under the conditions tested, vitamin E and GSH were unable to completely overcome negative effects of heat shock.

Effects of Supplemental Vitamin E and Selenium on Reproductive Function of Dairy Cows

While free radical formation and antioxidant status may be important for cellular function, including embryonic development, use of antioxidants to improve reproductive function in cattle has shown mixed results. The most widely-studied antioxidant therapy in cattle has been treatment with vitamin E and selenium. Vitamin E is the major lipid-soluble antioxidant in cells (Wang and Quinn, 2000) and selenium supplementation leads to an increase in activity of selenium-dependent glutathione peroxidases (Arthur, 2000). The primary reproductive benefit of supplementation with vitamin E and selenium is a reduced incidence of retained fetal membranes. In an early study, Trinder et al. (1969) showed that the injection of 680 IU of vitamin E and 15 mg of selenium one month before calving reduced the incidence of retained fetal membranes in animals on a selenium-deficient diet. This beneficial effect of vitamin E and selenium (Trinder et al., 1973; Julien et al., 1976a; Julien et al., 1976b; Segerson et al., 1981; Harrison et al., 1984; Eger et al., 1985; Aréchiga et al., 1994b) as well as selenium (Hemken et al., 1978; Reindhardt et al., 1978) was confirmed in several studies. In contrast, prepartum supplementation with vitamin E and selenium had no effect on incidence of retained fetal membranes in cows consuming diets adequate in selenium (Gwazdauskas et al., 1979; Schingoethe et al., 1982). Thus, some of the beneficial effects of vitamin E and selenium on retained fetal membranes are likely to be related to herd-to-herd variation in levels of feeding of these nutrients. Injection of selenium has also been reported to reduce the frequency of other reproductive disorders such as metritis and cystic ovaries (Harrison et al., 1984).

The effects of supplemental vitamin E and selenium on fertility are also controversial. Intramuscular injection of 680 IU vitamin E and 50 mg selenium 3 weeks before expected calving increased pregnancy rate to first service, reduced the number of services per conception and reduced the interval from calving to conception of dairy cows in Mexico (Aréchiga et al., 1994b). When the same dosage of vitamin E and selenium was injected 30 days postpartum, there was an increase in pregnancy rates to second service, a reduction on the number of services per conception and reduction on the interval from calving to conception (Aréchiga et al., 1998b). Similarly, Campbell and Miller (1998) found that supplemental vitamin E (1000 IU/day) during the dry period reduced interval from calving to first estrus. Not all studies have reported a beneficial effect of vitamin E and selenium on fertility, however. For example, injection of 680 IU vitamin E and 50 mg selenium (Kappel et al., 1984) or 2040 IU vitamin E (Hidiroglou et al., 1987) 3 weeks before expected calving had no effect on subsequent reproductive performance. The lack of consistent results among studies may be due to differences in vitamin E and selenium source, contents in diets, timing, dosage and methods of administration.

The mechanisms by which vitamin E and/or selenium affect reproductive function are unclear. There is evidence that vitamin E prevents lipid peroxidation during capacitation of bovine spermatozoa (O'Flaherty et al., 1997) and increases embryonic development to the blastocyst stage (Olson and Seidel Jr, 2000) while treatment of cows with vitamin E and selenium increase oocyte fertilization rate (Segerson et al., 1977) and stimulated uterine involution in cows with metritis (Harrison et al., 1986). Several lines of evidence indicate that vitamin E and/or selenium maintain the integrity of neutrophils. Neutrophils collected from cows fed a selenium-deficient diet had increased accumulation of hydrogen peroxide, decreased viability and reduced ability of intracellular kill of mastitis pathogens (Grasso et al., 1990). Vitamin E supplementation increased intracellular killing activity of bovine neutrophils without affecting neutrophil phagocytic index (Hogan et al., 1990; Hogan et al., 1992) and stimulated neutrophil chemotaxis (Politis et al., 1996). It has been suggested that the effect of vitamin E and selenium on prevention of retained fetal membranes is mediated by stimulating leukocyte activity and chemotaxis (Laven and Peters, 1996). Macrophages in placentomas of cows with retained fetal membranes had reduced activity of the lysozomal enzyme acid phosphatase (Miyoshi et al., 2002) as compared to macrophages from normally discharged placenta. There is evidence that metabolites from lipid peroxidation including conjugated dienes and hydroperoxides are higher in retained placental samples as compared to non-retained placenta controls (Kankofer, 2001).

According to the National Research Council (NRC, 1989), the vitamin E requirement for dairy cows is 15 IU/kg of dry matter intake. This value corresponds to 150 and 300 IU/day of vitamin E for dry and lactating cows, respectively. This dietary requirement was determined based on the ability of vitamin E to prevent signs of nutritional muscular dystrophy. However, higher amounts of vitamin E are required to maintain mammary health and reproductive efficiency (Smith et al., 1997). Recent studies recommend feeding at least 500 IU/day of supplemental vitamin E for lactating cows and 1000 IU/day for dry cows (Weiss, 1988; Smith et al., 1997). Plasma concentrations of α-tocopherol greater than 3.5-4.0 μg/ml are considered adequate as demonstrated by the ability of neutrophils to exert intracellular killing of bacteria (Hogan et al., 1993).

Antioxidant Therapy During Heat Stress

Antioxidants have been used in an attempt to improve fertility of heat stressed cows. Studies indicated that short-term supplementation with vitamin E or β-carotene had no beneficial effect on reproductive function. Administration of 3000 IU vitamin E at the time of artificial insemination did not consistently improve pregnancy rates during summer or winter in Florida (Ealy et al., 1994). Similarly administration of β-carotene at -6, -3 days before anticipated date of artificial insemination and at the time of artificial insemination (Aréchiga et al., 1998b) or supplemental β-carotene on the ration for 15-89 days before artificial insemination had no effect pregnancy rates (Aréchiga et al., 1998a). In contrast, long-term supplementation with β-carotene on the ration for≥ 90 days before artificial insemination increased pregnancy rates at 120 days postpartum from 21.1% in control to 35.4 % in β-carotene supplemented cows (Aréchiga et al., 1998a).

Genotype

Breeds of cattle have evolved to be adapted for different environments. It is well known that *Bos indicus* and certain *B. taurus* breeds such as Senepol (tropically adapted *B. taurus*) are more resistant to elevated temperature and humidity than breeds that evolved in Europe such as Angus and Holstein. This difference extends to effects of heat stress on reproduction. For example, the average depression on fertility (measured as success or failure in producing a live calf) associated with heat stress was higher for *B. taurus* than *B. indicus* breeds (Turner, 1982).

Recently, a study was conducted using ultrasound-guided follicular aspiration for collection of Holstein and Brahman oocytes during the cool and hot season. The percentage of Holstein oocytes with normal morphology decreased from 80% in the cool season to 25% in the hot season. The percentage of in vitro fertilized oocytes that developed to the 8-cell, morula and blastocyst stage was higher in the cool season (44.4% 8-cell, 34.2% morula and 29% blastocyts) than the hot season (1.1% 8-cell, 0% morula and 0% blastocyst) (Rocha et al., 1998). In contrast to the sensitivity of Holstein oocytes to heat stress, there was no effect of season on the proportion of Brahman oocytes with normal morphology or that developed to the 2-cell, 8-cell, morula or blastocyst stage (Rocha et al., 1998). This result indicates that either 1) the thermoregulatory ability of Brahman cows reduced maternal hyperthermia as compared to *B. taurus*, 2) oocytes from Brahman cows contain intrinsic mechanisms that allow them to survive after exposure to elevated temperature or 3) some combination of both mechanisms.

Certainly much of the genetic variation in resistance to heat stress results from the ability of thermotolerant breeds to regulate body temperature (Adeyemo et al., 1979; Bennett et al., 1985; Hammond et al., 1996; Hammond et al., 1998; Gaughan et al., 1999). In addition, however, there are indications that breed differences in thermal resistance extend to the cellular level. In one study, the lethal effects of a severe heat shock of 45°C for 3 h on viability of lymphocytes was greater for lymphocytes from Angus heifers than for lymphocytes for Brahman and Senepol heifers. When examining effects of 42° C, exposure of phytohemaglutinin-treated lymphocytes for 12 h caused a greater reduction in viability for Angus lymphocytes than for Brahman or Senepol lymphocytes. In contrast, 42°C for 12 h reduced proliferation of phytohemaglutinin-treated lymphocytes to the same degree regardless of breed (Kamwanja et al., 1994).

Breed differences in resistance to elevated temperature also were observed in reproductive tract tissues. Exposure to 43°C increased secretion of nondialyzable [^{3}H]-labeled macromolecules by cultured oviductal tissues from both oviducts of Brahman cows and from the oviduct contralateral to the side of ovulation of Holstein cows but decreased secretion by tissue from oviduct ipsilateral to ovulation of Holstein cows. Heat shock of 43°C also caused an increase in [^{3}H]thymidine incorporation by endometrial explants from Brahman cows while suppressing incorporation in explants from Holstein cows (Malayer and Hansen, 1990a).

The molecular basis for genetic differences in cellular resistance to elevated temperatures in *B. indicus* and Senepol cattle are not known. It is possible that cells from *B. indicus* cows have more HSP70 and therefore are better able to stabilize and refold damaged proteins (Hartl and Martin, 1992). However, while heat shock increased intracellular amounts of HSP70 in lymphocytes (Kamwanja et al., 1994) and endometrial tissue (Malayer and Hansen, 1990a) these responses was not altered by breed.

CHAPTER 3
HEAT-SHOCK INDUCED APOPTOSIS IN PREIMPLANTATION BOVINE EMBRYOS IS A DEVELOPMENTALLY-REGULATED PHENOMENON

Introduction

Preimplantation embryonic development is a dynamic process which involves cell proliferation, differentiation and death. Those processes are tightly regulated by signaling between the embryo and the maternal environment. The ability of the embryo to respond to changes in its environment is limited during the first cleavage divisions when most of the embryonic genome is still inactive (Memili and First, 2000) and when systems for regulation of osmotic balance are not completely functional (Lane, 2001; Van Winkle, 2001). This period of low transcriptional activity creates a window in which embryos are particularly sensitive to certain forms of stress. One of the alterations in the maternal environment causing profound effects on embryonic survival is an increase in body temperature due to heat stress or fever. Exposure of embryos to elevated temperature decreases development (Aréchiga et al., 1995; Edwards and Hansen, 1997) and reduces protein synthesis (Edwards et al., 1995). Indeed, exposure of females to heat stress during preimplantation development reduces embryo survival (Dutt, 1963; Tompkins et al., 1967; Putney et al., 1988a; Ealy et al., 1993).

In several species, the deleterious effects of heat shock decrease as embryos advance in development. This has been shown in vivo in sheep (Dutt, 1963), pigs (Tompkins et al., 1967), cattle (Putney et al., 1988a) and rabbits (Wolfenson and Blum, 1988) and in vitro for cattle (Ealy et al., 1995; Edwards and Hansen, 1997). Thus, the embryo acquires one or more thermoprotective responses as embryonic development proceeds.

One of the processes that may be involved in developmental acquisition of resistance to heat shock may be stress-induced apoptosis. Apoptosis plays a role in mammalian development as a quality control mechanism to eliminate cells that are damaged, nonfunctional, abnormal or misplaced (Jacobson et al., 1997; Meier et al., 2000; Beere and Green, 2001). Cells severely damaged by stress that do not undergo apoptosis often become necrotic (Fuse et al., 1998; Gorman et al., 1999).

TUNEL-positive embryos have been demonstrated in mouse (Brison and Schultz, 1997; Brison and Schultz, 1998; O'Neill, 1998), human (Yang et al., 1998; Hardy, 1999), and bovine (Byrne et al., 1999; Matwee et al., 2000; Neuber et al., 2000) species. The occurrence of apoptosis in bovine embryos as determined by TUNEL staining has been shown to be developmentally regulated. Spontaneous apoptosis was first observed in bovine embryos at the 8-16 cell stage (Byrne et al., 1999; Matwee et al., 2000). This stage of development in cattle is coincident with the time of the major activation of the embryonic genome (Memili and First, 2000) and it is possible therefore that embryonic transcription is involved in apoptotic responses in embryos.

While apoptosis is known to occur in preimplantation embryos, there are few studies on extrinsic or intrinsic control systems for activation of apoptosis in preimplantation embryos or the ontogeny of such systems. Moreover, it is not known whether agents like heat shock, which can induce apoptosis in many cell lines through activation of the enzyme sphingomyelinase (Verheij et al., 1996; Haimovitz-Friedman et al., 1997; Pena et al., 1997), also induce apoptosis in preimplantation embryos. The working hypothesis of this series of experiments was that heat-induced apoptosis is a developmentally-regulated process. Experiments were performed to: 1) determine whether heat shock can induce apoptosis in preimplantation embryos; 2) test whether heat-induced apoptosis in embryos depends upon stage of development; 3) evaluate whether heat-shock induced changes in caspase activity parallel patterns of apoptosis; and 4) ascertain whether exposure to a mild heat shock can protect embryos from heat-induced apoptosis. The last objective was conducted because of evidence that apoptosis in heat shocked cells can be reduced by HSP70 (Mosser et al., 1997; McMillan et al., 1998) or by a mild heat shock that presumably induces HSP70 synthesis (Boreham et al., 1997; Buzzard et al., 1998; McMillan et al., 1998).

Materials and Methods

Materials

Essentially fatty-acid free bovine serum albumin (BSA) was purchased from Sigma Chemical Company (St. Louis, MO). Bovine steer serum was from Pel-Freez (Rogers, AR). Modified Tyrode's solutions were obtained from Cell and Molecular Technologies (Lavallette, NJ) to prepare HEPES-Tyrode's Albumin Lactate Pyruvate (TALP), in vitro fertilization (IVF)-TALP and Sp-TALP (Parrish et al., 1986). Pituitary-derived follicle stimulating hormone (FSH; Folltropin®-V) was purchased from Vetrepharm Canada Inc. (London, Ontario). Percoll was from Amersham Pharmacia Biotech (Uppsala, Sweden). Frozen semen from various bulls was purchased from American Breeders Service (Madison, WI) or donated by Select Sires Inc. (Rocky Mount, Virginia). Potassium Simplex Optimized Medium (KSOM) was obtained from Cell and Molecular Technologies. The KSOM, which contains 1 mg/ml BSA, was modified on the day of use by adding an additional 2 mg/ml EFAF-BSA, 2.5 μg/ml gentamicin, essential amino acids (Basal Medium Eagle) and non-essential amino acids (Minimum Essential Medium) purchased from Sigma Chemical Company (St. Louis, MO). Oocyte collection medium was Tissue Culture Medium 199 (TCM-199) with Hank's salts without phenol red and supplemented with 2% (v/v) bovine steer serum (containing 2 U/ml heparin), 100 U/ml penicillin-G, 0.1 mg/ml streptomycin, and 1 mM glutamine. Oocyte maturation medium was TCM-199 with Earle's salts supplemented with 10% (v/v) steer serum, 22 μg/ml sodium pyruvate, 20 μg/ml FSH, 2 μg/ml estradiol 17-β, 50 μg/ml gentamicin and an additional 1 mM glutamine.

In Situ Cell Death Detection Kit (fluorescein) and propidium iodide were obtained from Roche Diagnostics Corporation (Indianapolis, IN) and Sigma Chemical Company (St. Louis, MO), respectively. Polyvinylpyrrolidone (PVP) was purchased from Eastman Kodak Company (Rochester, NY). Prolong Antifade Kit was obtained from Molecular Probes (Eugene, Oregon), RQ1 RNA-free DNase was from Promega (Madison, WI) and RNase A was from Qiagen (Valencia, CA). PhiPhiLux-G_1D_2 was obtained from OncoImmunin, Inc. (Gaithersburg, MD). Other reagents were purchased from Fisher (Pittsburgh, PA) or Sigma Chemical Company (St. Louis, MO).

In Vitro Production of Embryos

Embryos were produced using procedures described earlier (Paula-Lopes et al., 1998; Rivera and Hansen, 2001) except that culture medium for embryos was different. Briefly, cumulus-oocyte complexes (COCs) were obtained by slicing 2-10 mm follicles on the surface of the ovaries obtained from slaughtered cows (mixture of beef and dairy cattle). Cumulus-oocyte complexes which had at least one layer of compact cumulus cells were washed two times and used for subsequent steps. Groups of 10 COCs were placed in 50 µl drops of oocyte maturation medium overlaid with mineral oil. For most experiments, COCs were matured for 22 h at 38.5°C in an atmosphere of 5% (v/v) CO_2 in humidified air. A longer time for oocyte maturation (24-26 h) was used for the experiment with caspase activity for technical reasons to ensure that the fluorescence microscope required was available when needed. Cumulus-oocyte complexes were removed from maturation drops and washed one time in HEPES-TALP. For in vitro fertilization, groups of 30 COCs were transferred to four well plates containing 600 µl IVF-TALP and 25 µl PHE [0.5 mM penicillamine, 0.25 mM hypotaurine and 25 µM epinephrine in 0.9% (w/v) NaCl] per well and fertilized with ~1 x 10^6 Percoll-purified spermatozoa. After 8-10 h at 38.5°C and 5% (v/v) CO_2 in humidified air, presumptive zygotes were removed from fertilization wells, denuded of cumulus cells by vortexing in 30 µl HEPES-TALP for 5 min in a microcentrifuge tube, washed 2-3 times in HEPES-TALP and placed in groups of 25-30 in 50 µl drops of modified KSOM overlaid with mineral oil at 38.5°C and 5% CO_2 (v/v) in humidified air. Embryos were harvested from culture at different times according to the specific experimental design. Two-cell embryos were harvested at 28-30 h after insemination; 4-cell embryos at 37-39 h; 8-16 cell embryos on day 3 or 4 after insemination and embryos $\geq$ 16 cells on day 5 after insemination.

Using this system of embryo production, cleavage rate ranged from 77-100% across all the experiments. In several replicates, one plate of embryos was not subjected to treatment or any staining procedure and embryos were allowed to develop to the blastocyst stage to verify adequacy of culture conditions. In these plates, the percentage of putative zygotes developing to blastocyst by day 8 after insemination ranged from 33-59%.

TUNEL and Propidium Iodide Labeling

The TUNEL procedure was used to detect DNA fragmentation observed in late stages of apoptosis. The enzyme terminal deoxynucleotidyl transferase is a DNA polymerase that catalyzes the transfer of a fluorescein isothiocyanate-conjugated dUTP nucleotide to a free 3' hydroxyl group present in DNA strand breaks. Embryos were removed from culture medium (modified KSOM) and washed 4 times in 100 µl drop of PBS (pH 7.4) containing 1 mg/ml polyvinylpyrrolidone (PBS-PVP) by transferring the embryos from drop to drop. Zona pellucida-intact embryos were fixed in a 100 µl drop of 4% (w/v) paraformaldehyde in PBS, pH 7.4 for 1 h at room temperature, washed twice in PBS-PVP, transferred to a poly-l-lysine coated slide and allowed to dry for 24 h at room temperature. Embryos were then washed twice by dipping the slide in a Coplin jar containing PBS-PVP (2 min/wash) and permeabilized in 0.5% (v/v) Triton X-100 containing 0.1% (w/v) sodium citrate for 1 h at room temperature. Positive controls were incubated with RQ1 RNase-free DNase (50 U/ml) at 37°C for 1 h. Embryos were washed in PBS-PVP and incubated with 50 µl TUNEL reaction mixture (containing fluorescein isothiocyanate-conjugated dUTP and the enzyme terminal deoxynucleotidyl transferase as prepared by the manufacturer) for 1 h at 37°C in the dark. Negative controls were incubated in the absence of terminal deoxynucleotidyl transferase. Embryos were then incubated with RNase A (50 µg/ml) for 1 h at room temperature, followed by 0.5 µg/ml propidium iodide for 30 min at room temperature. Embryos were washed 4 times in PBS-PVP to remove excess propidium iodide. A coverslip was mounted with 16 µl mounting medium containing Antifade as recommended by the manufacturer. Labeling was observed using a Zeiss Axioplan 2 fluorescence microscope with dual filter. Each embryo was analyzed for total number of nuclei and number of TUNEL-labeled nuclei. Some embryos were also examined for photomicroscopy using a Bio-Rad 1024ES laser scanning confocal microscope. For fluorescein, an argon ion laser adjusted to below 560 nm was used, and for propidium iodide, a helium-neon laser adjusted to above 560 nm was used. Images were obtained using a 30X objective, 10% power and Z-steps of 0.5-1.0 μm.

Caspase Activity

PhiPhiLux-G_1D_2 is a fluoroprobe that incorporates the group II caspase-recognition sequence DEVD into a bifluorophore-derivitized peptide that mimics the structural loop conformation present in native protease cleavage sites. Group II caspases include caspase 3, caspase 2 and caspase 7. In this molecule, the core peptide, GDEVDGI, is coupled to a molecule of rhodamine on each side of the cleavage site. The two rhodamines interact as a dimer and emit a stable blue-green fluorescence. Cleavage of the substrate disrupts this interaction between rhodamine moieties to result in enhanced green fluorescence (excitation peak 490 nm and emition peak 520).

To measure caspase activity, embryos were removed from culture medium and washed 3 times in 50 μl drops of prewarmed HEPES-TALP. Embryos were incubated in 25 μl microdrops of HEPES-TALP containing 5 μM PhiPhiLux-G_1D_2 at 39°C for 40 min in the dark. Negative controls were incubated in HEPES-TALP only. Following incubation, embryos were washed twice in 50 μl drops of HEPES-TALP and placed on poly-l-lysine coated slides and mounted with a coverslip. Caspase activity was determined immediately after the end of heat shock using a Zeiss Axioplan 2 fluorescence microscope with a 45X objective. Images were obtained using a Spot camera and software (Diagnostic Instruments, Inc.). Pictures were taken using a background subtract feature from the spot software and digital images were stored as .tiff files. Fluorescence intensity was analyzed using IPLab for MacIntosh version 3.5 (BioVision Technologies, Inc., Exton, PA). Each embryo represented a region of interest. Using the computer mouse, a circular draw function was manually performed for each region of interest and the pixel intensity per unit area was determined.

Experiments

A series of six experiments were conducted to evaluate heat-induced apoptosis. For TUNEL analysis, embryos were collected at different times in development, transferred to a new drop of modified KSOM (15-30 embryos per drop; within a run, similar numbers of embryos per drop were used for each treatment) and maintained at 38.5° C for 24 h or exposed to various heat shock (40, 41 or 42°C) of 3-9 h duration. A heat shock of 42°C was used in the initial experiment to maximize probability of inducing of apoptosis. In subsequent experiments, embryos were exposed to more physiological temperatures (40-41°C) similar to those experienced by heat-stressed cows. Heat-shocked embryos were then returned to 38.5°C. At 24 h after the initiation of heat shock, embryos were fixed in 4% (w/v) paraformaldehyde, transferred to a poly-l-lysine coated slide and saved at 4°C until analysis by TUNEL. For caspase activity, embryos were exposed to either 38.5°C or 41°C for 9 h. Immediately after the end of heat shock caspase activity was determined as previously described. Details of specific experiments are described below.

Heat-induced apoptosis in bovine embryos ≥ 16 cell stage

Bovine embryos that were ≥ 16 cells were collected on day 5 after insemination and transferred to a new drop of modified KSOM. Embryos were cultured at 38.5°C for 24 h or were heat shocked at 41 or 42°C for 9 h followed by 38.5°C for 15 h. Analysis by TUNEL was then performed. The experiment was replicated 4 times using 208 embryos (50–85 embryo/group).

Effect of magnitude and duration of heat shock on heat-induced apoptosis

The purpose of this experiment was to determine whether the degree of apoptosis was proportional to the severity of heat shock. This was accomplished by heat shocking embryos at two different temperatures for variable durations. Embryos ≥ 16 cells were collected on day 5 after insemination and transferred to a new drop of modified KSOM. Embryos were cultured at either 38.5°C for 24 h or were heat shocked at 40 or 41°C for 3, 6 or 9 h followed by 38.5°C for a total culture period of 24 h. Twenty-four hours after the initiation of heat-shock, embryos were fixed in 4% paraformaldehyde, transferred to a poly-l-lysine coated slide and saved at 4°C until analysis by TUNEL. The experiment was replicated 4 times using 342 embryos (30-70 embryos/group).

Heat-induced apoptosis in bovine embryos at the 2- or 4-cell stage

Embryos at the 2- or 4-cell stage were collected at 28-30 and 37-39 h after insemination, respectively. Embryos were transferred to a new drop of modified KSOM and cultured at 38.5°C for 24 h or were heat shocked at 41°C for 9 h followed by 38.5°C for 15 h. Embryos were fixed in 4% paraformaldehyde, transferred to a poly-l-lysine coated slide and saved at 4°C until analysis by TUNEL. The experiment was replicated 7 times using 491 embryos (94-146 embryos/group).

Heat-induced apoptosis in bovine embryos at the 8-16 cell stage on day 3 after insemination

Embryos at the 8-16 cell stage were collected on day 3 after insemination and transferred to a new drop of modified KSOM. Embryos were then cultured at 38.5°C for 24 h or were heat shocked at 40 or 41°C for 9 h followed by 38.5°C for 15 h. At the end of culture, embryos were fixed and subjected to TUNEL analysis. The experiment was replicated 3 times using 188 embryos (52-64 embryos/group).

Induced thermotolerance in bovine embryos at the 8-16 cell stage on day 4 after insemination

Embryos at the 8-16 cell stage were collected on day 4 after insemination and transferred to a new drop of modified KSOM. Embryos were then subjected to four treatments: control (38.5°C for 24 h), mild heat shock (40°C for 80 min followed by 38.5°C), severe heat shock (41°C for 9 h followed by 38.5°C for 15 h) or thermotolerance (40°C for 80 min followed by 2 h at 38.5°C and 9 h at 41°C). At 24 h after initiation of temperature treatment, embryos were fixed in 4% paraformaldehyde, transferred to a poly-l-lysine coated slide and saved at 4°C until analysis by TUNEL. The experiment was replicated 4 times using 473 embryos (111-120 embryos/group).

Caspase activity in bovine embryos

Embryos at the 2-cell stage or ≥ 16-cell stage were collected 28-30 h after insemination or on day 5 after insemination, respectively. Embryos were transferred to a new drop of modified KSOM and cultured at 38.5°C for 24 h or heat shocked at 41°C for 9 h followed by 38.5°C for 15 h. After heat shock, embryos were washed 3 times in a 50 μl drop of prewarmed HEPES-TALP and analyzed for caspase activity as described above. The experiment was replicated 5 times using 350 embryos. Caspase activity was quantified using 135 embryos produced in two replicates (22-42 embryos/group).

Statistical Analysis

Data were analyzed by least-squares analysis of variance using the General Linear Models (GLM) procedure of SAS (SAS, 1989). Percentage data were transformed using the arcsin transformation before analysis. Dependent variables were total cell number, percentage of apoptotic cells and the arcsin of the percentage of cells displaying apoptosis. Independent variables varied according to the experimental design and included treatment, day (i.e., replicate), and stage of embryonic development. The mathematical model included main effects and all interactions. All main effects were considered fixed. Orthogonal contrasts and a means separation procedure of SAS called pdiff were performed when appropriate to determine differences between levels of individual treatments.

Results

Heat-Induced Apoptosis in Embryos ≥ 16-Cells

Figure 3-1A-C displays representative confocal digital images of embryos that were collected on day 5 after insemination (if cell number was ≥ 16 cells), cultured at either 38.5°C for 24 h or at 41°C for 9 h and 38.5°C for 15 h, and then subjected to the TUNEL reaction. An embryo exposed to 38.5°C is shown in Figure 3-1A while embryos exposed to 41°C are shown in Figure 3-1B-C. An increase in the proportion of nuclei labeling positive for TUNEL (yellow in color) is apparent in the embryos exposed to 41°C. The percentage of cells undergoing apoptosis following heat shock was determined in several experiments. In the first experiment, exposure of embryos ≥ 16 cells to 41 or 42°C for 9 h increased ($p < 0.05$) the percentage of cells undergoing apoptosis 24 h after initiation of heat shock as compared to embryos cultured at 38.5°C for 24 h (Figure 3-2A). Heat shock also tended to reduce ($p = 0.07$) total number of cells per embryo 24 h after initiation of treatment (Figure 3-2B). The effect of heat shock was similar for 41°C or 42°C.

In a second experiment, embryos were exposed to temperatures more characteristic of rectal temperatures experienced by cows during heat stress. In addition, the effect of different durations of heat shock were examined. Exposure to either 40°C ($p < 0.05$) or 41°C ($p < 0.001$) increased percentage of cells that were apoptotic as compared to 38.5°C (Figure 3-3A). When embryos were subjected to 40°C, there was a quadratic ($p < 0.01$) effect of time on the percentage of cells that were apoptotic. Percent apoptosis was higher for embryos at 40°C than for embryos at 38.5°C but the percent apoptosis at 40°C was similar for durations of 3, 6 and 9 h. The effect of time on the induction of apoptosis was even more pronounced at 41°C. Again there was a quadratic ($p < 0.001$) increase in the percentage of apoptotic cells as time of exposure increased. However, neither 40°C nor 41°C affected the total number of cells per embryo (Figure 3-3B).

Lack of Heat-Induced Apoptosis in Bovine Embryos at the 2- or 4-Cell Stage

Representative confocal digital images of embryos assessed by TUNEL for apoptosis 24 h after collection at the 2-cell stage are shown in Figure 3-1D (embryo at 38.5°C continuously) and Figure 3-1E and F (embryos exposed to 41°C for 9 h and 38.5°C for 15 h before TUNEL labeling). Note the absence of TUNEL labeling in all embryos and the increased incidence of nuclear fragmentation and reduced cell number in heat-shocked embryos.

When data were analyzed, exposure of 2- or 4-cell embryos to 41°C for 9 h had no effect on the percentage of cells undergoing apoptosis 24 h after initiation of heat shock when compared to embryos cultured continuously at 38.5°C. Indeed, the proportion of apoptotic cells at these stages of embryonic development was very low and ranged from 0.04-0.66% (Figure 3-4A). This compares to 7.8-8.0% apoptosis in $\geq$ 16-cell embryos collected at day 5 and cultured at 38.5°C (Figure 3-2A and Figure 3-3A). While not affecting apoptosis, heat shock at the 2- (38.5 vs 41°C, $p< 0.001$) and 4-cell stage (38.5 vs 41°C, $p< 0.001$) reduced total embryo cell number 24 h after initiation of treatment (Figure 3-4B).

Heat-induced Apoptosis and Induced Thermotolerance at the 8-16 Cell Stage

Two experiments were conducted to determine whether apoptosis occurs at the 8-16 cell stage in response to heat shock. In the first study, exposure of day 3 embryos at the 8-16 cell stage to 40°C or 41°C for 9 h had no effect on the percentage of apoptotic cells (Figure 3-5A) or on total embryo cell number (Figure 3-5B) at 24 h after initiation of temperature treatments. In the second experiment (Figure 3-6), 8-16 cell embryos were collected on day 4 rather than day 3. In addition it was tested whether exposure to a mild heat shock of 40°C for 80 min would make embryos more resistant to apoptosis induced by a severe heat shock of 41°C for 9 h. Results are shown in Figure 3-6. There was no difference between control embryos at 38.5°C and embryos subjected to 40°C for 80 min on percentage of cells undergoing apoptosis or embryo cell number (Figure 3-6). In contrast, exposure to 41°C for 9 h increased (38.5 vs 41°C, $p< 0.001$) the proportion of cells that were apoptotic 24 h after initiation of temperature treatments and reduced (38.5 vs 41°C, $p < 0.01$) embryo cell number. Preincubation at 40°C for 80 min blocked (40/41°C vs 41°C, $p < 0.001$) heat-induced apoptosis, however. Embryo cell number remained lower (38.5 vs 40/41°C, $p < 0.001$) for heat shocked embryos than controls, however, regardless of whether embryos were preincubated at 40°C (Figure 3-6B). Thus, there was induction of thermotolerance with respect to apoptosis but not cell number because preincubation at 40°C for 80 min didn't prevent the reduction in cell number caused by 41°C.

Caspase Activity

Representative digital images illustrating caspase activity in 2 cell embryos and embryos $\geq$ 16 cell stage are shown in Figure 3-7. Note the higher caspase activity for day 5 embryos $\geq$ 16 cell stage exposed to heat shock of 41°C for 9 h (Figure 3-7D-F) when compared to embryos exposed to 38.5°C (Figure 3-7C). However, when 2-cell embryos were exposed to the same heat shock treatment there was no induction of caspase activity (Figure 3-7A-B)

The effect of heat shock (41°C for 9 h) on caspase activity was dependent upon stage of development (stage x temperature, $p < 0.01$). Heat shock increased (38.5 vs 41°C, $p < 0.001$) caspase activity for day 5 bovine embryos (Figure 3-8) as compared to 38.5°C control. In contrast, caspase activity of two-cell embryos was not affected by heat shock (Figure 3-8).

Discussion

One function of apoptosis is to eliminate cells damaged by stress. Heat shock, for example, induces apoptosis in many cell types (Boreham et al., 1997; Matsumoto et al.,

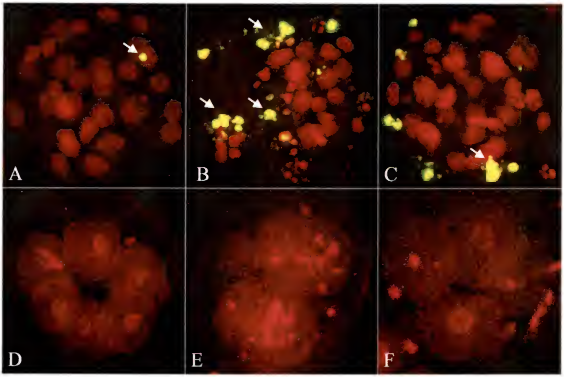

Figure 3-1. Representative confocal images illustrating the frequency of apoptotic nuclei in bovine embryos subjected to TUNEL analysis 24 h after initiation of temperature treatment. Embryos were labeled with fluorescein isothiocyanate-conjugated dUTP (green channel) and propidium iodide (red channel). Each panel is a display of a single embryo. Panels A (treated at the ≥ 16-cell stage on day 5 after insemination) and D (treated at the 2-cell stage) are embryos cultured at 38.5°C for 24 h and then subjected to TUNEL. Panels B and C (treated at the ≥ 16-cell stage) and panels E and F (treated at the 2-cell stage) are embryos exposed to a heat shock of 41°C for 9 h followed by 38.5°C for 15 h before analysis by TUNEL. Arrows point to apoptotic nuclei. Nuclei labeled with TUNEL were frequently fragmented. Note that day 5 embryos exposed to 41°C for 9 h displayed a higher frequency of TUNEL-positive cells as compared to embryos exposed to 38.5°C. In contrast, 2-cell embryos exposed to 41°C for 9 h did not exhibit apoptosis (Figure 3-1E-F). Heat-shocked 2-cell embryos exhibited nuclear fragmentation and had lower cell number as compared to embryos cultured at 38.5°C. (Note that the fact that embryos in panels E and F were greater than 2-cell in number at the time of TUNEL analysis reflects cleavage following initiation of treatment).

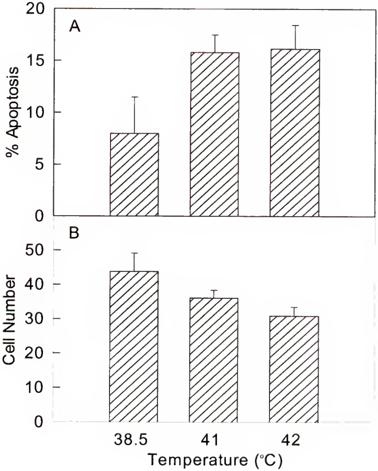

Figure 3-2. Heat-induced apoptosis in bovine embryos (≥ 16-cells) treated on day 5 after insemination. The experiment was replicated 4 times using 50–85 embryos/group. Results are least-squares means ± SEM. As compared to embryos cultured at 38.5°C continuously for 24 h, heat shock of 41 or 42°C for 9 h increased ($p < 0.05$) percentage of cells undergoing apoptosis at 24 h after initiation of heat shock and tended to reduce ($p = 0.07$) the total number of cells per embryo at this time. There was no significant difference between 41 or 42°C.

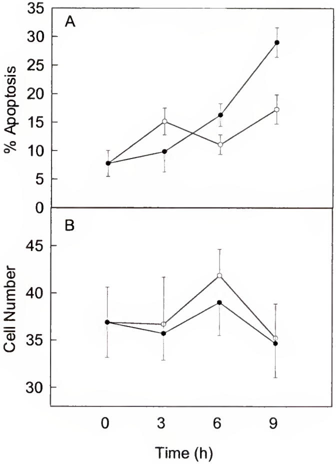

Figure 3-3. Time dependence of heat-induced apoptosis in bovine embryos (≥ 16-cells) treated on day 5 after insemination and assessed by TUNEL 24 h after initiation of heat shock. The experiment was replicated 4 times using 30-70 embryos/group. Results are least-squares means ± SEM. Zero hours represents embryos cultured at 38.5°C for 24 h. Heat shock of 40°C ($p < 0.05$) (open circles) and 41°C ($p < 0.001$) (closed circle) increased percentage of cells undergoing apoptosis at 24 h after initiation of heat shock. There was a quadratic effect of time at 40°C ($p < 0.01$) and 41°C ($p < 0.001$). Heat shock had no effect on total cell number per embryo.

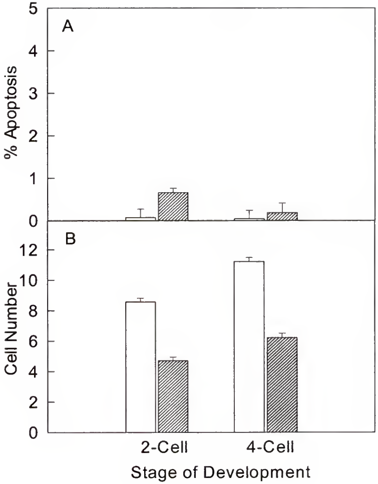

Figure 3-4. Lack of heat-induced apoptosis in bovine embryos treated at the 2 or 4-cell stage and assessed by TUNEL 24 h after initiation of heat shock. The experiment was replicated 7 times using 94-146 embryos/group. Results are least-squares means ± SEM. Open bars are control embryos and hatched bars are embryos exposed to heat shock. Heat shock of 41°C for 9 h at the 2- or 4-cell stage had no effect on the percentage of cells undergoing apoptosis at 24 h after initiation of heat shock. However total number of cells per embryo at 24 h after initiation of treatment was reduced by heat shock at the 2- (38.5 vs 41°C, $p < 0.001$) and 4- (38.5 vs 41°C, $p < 0.001$) cell stage.

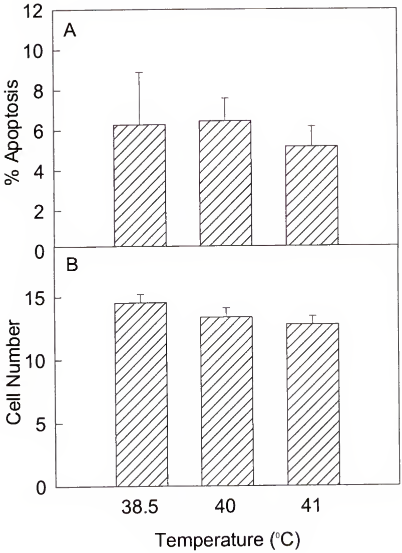

Figure 3-5. Lack of heat-induced apoptosis in 8-16 cell bovine embryos treated on day 3 after insemination and assessed by TUNEL 24 h after initiation of heat shock. The experiment was replicated 3 times using 52-64 embryos/group. Results are least-squares means ± SEM. Heat shock of 40°C or 41°C for 9 h had no significant effect on subsequent percentage of cells undergoing apoptosis or total cell number per embryo.

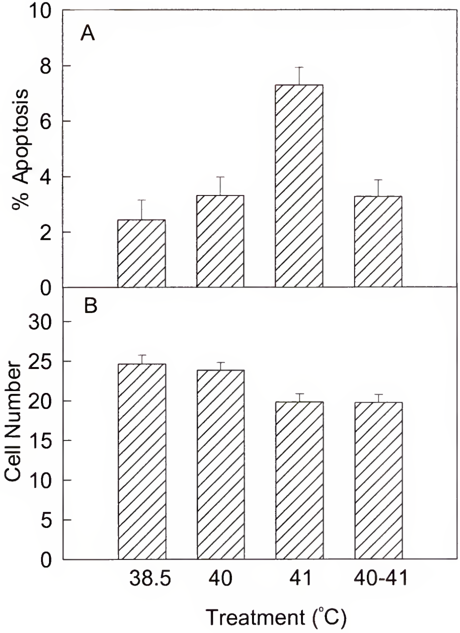

Figure 3-6. Induced thermotolerance in bovine embryos at the 8-16 cell stage treated on day 4 after insemination and assessed by TUNEL 24 h after initiation of heat shock. The experiment was replicated 4 times using 111-120 embryos/group Results are least-squares means ± SEM. Exposure of embryos to a mild heat shock of (40°C for 80 min) blocked apoptosis induced by exposure to a subsequent heat shock of 41°C for 9 h (40/41°C vs 41°C, $p< 0.001$). However, pre-exposure to 40°C did not prevent the subsequent reduction in total embryo cell number induced by 41°C for 9 h (38.5°C vs 40/41°C, $p< 0.05$).

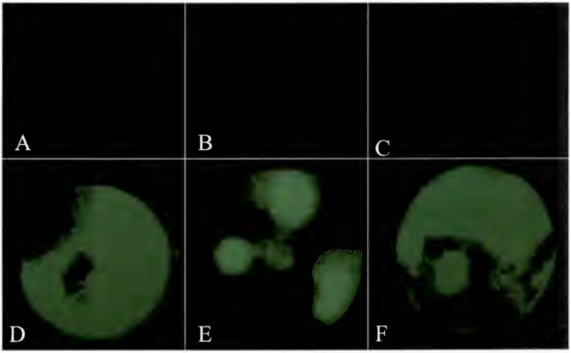

Figure 3-7. Representative digital images illustrating group II caspase activity in embryos ≥ 16 cell stage and 2 cell embryos. Panels A (2-cell embryo) and C (≥ 16-cell embryo) are embryos cultured at 38.5°C. Panel B (2-cell embryo) and panels D, E and F (≥ 16-cell embryo) are embryos exposed to 41°C for 9 h. Caspase activity was determined immediately after the end of the heat shock period. Note that day 5 embryos ≥ 16 cell stage exposed to heat shock of 41°C for 9 h had higher caspase activity when compared to embryos exposed to 38.5°C. However, exposure of 2 cell embryos to the same heat shock did not increase caspase activity.

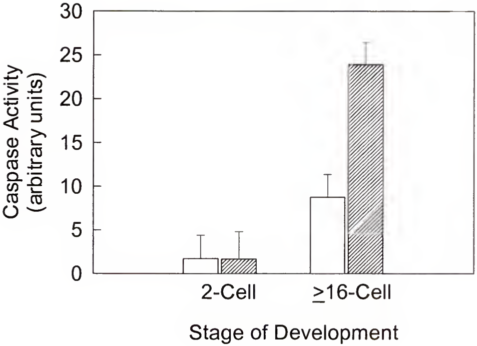

Figure 3-8. Group II caspase activity in bovine embryos. Results are least-squares means ± SEM of data from 135 embryos (22-42/group) in a total of two replicates. Open bars are control embryos and hatched bars are embryos exposed to heat shock. Exposure of embryos to 41°C for 9 h did not increase caspase activity of 2-cell embryos but did increase caspase activity of embryos ≥ 16-cells on day 5 after insemination (stage x temperature, $p < 0.01$).

1997; Mosser et al., 1997; Fuse et al., 1998; McMillan et al., 1998). While several recent studies have demonstrated that preimplantation embryos undergo apoptosis in a stage-specific manner (Byrne et al., 1999; Matwee et al., 2000), few studies have evaluated the role of stress in induction of apoptosis in embryos. The present study demonstrated that heat shock, which is a cellular stress associated with embryonic loss in vivo (Dutt, 1963; Tompkins et al., 1967; Putney et al., 1988a; Ealy et al., 1993) and in vitro (Ealy et al., 1995; Edwards and Hansen, 1997; Ju et al., 1999), can induce apoptosis as determined by TUNEL reaction. Nuclei could conceivably be TUNEL-positive because of necrosis or because of DNA strand breaks that occur as artefacts of sample preparation and fixation. However, TUNEL labeling of apoptotic nuclei is confined to the nuclei, as seen in the current study, while labeling of necrotic cells can spread throughout the cytoplasm (Hardy, 1999). Further evidence that the TUNEL-positive blastomeres in the present study represent apoptosis was the observation of nuclear fragmentation characteristic of apoptosis and increased activity of group II caspases.

The temperatures that induced apoptosis included those characteristic of heat-stressed cows (40 and 41°C), suggesting apoptosis could be induced in preimplantation embryos exposed to maternal hyperthermia. Moreover, the degree of apoptosis experienced by heat-shocked embryos generally reflected the severity of heat shock. The percent of cells positive for apoptosis increased with increasing heat shock temperatures and, at least at 41°C, increased with duration of heat shock. At 40°C also, induction of apoptosis was time dependent, with no increase in apoptosis seen after an exposure of 80 min (Figure 3-6) but an increase in percent apoptotic cells after 3, 6 or 9 h exposure (Figure 3-3). The observation that the degree of apoptosis following exposure to 40°C for 3, 6 and 9 h was generally similar could possibly reflect time-dependent cellular adaptation to prevent apoptosis following prolonged exposure to a mild heat shock of 40°C that was not possible for a more severe heat shock of 41°C.

Acquisition of heat-induced apoptosis is developmentally regulated and does not occur until approximately day 4 after insemination at the 8-16 cell stage. Spontaneous apoptosis in bovine embryos is first observed also at the 8-16 cell stage (Matwee et al., 2000). The failure to observe apoptosis in 8-16 cell embryos collected at day 3 after insemination may mean that either 1) development or apoptosis mechanisms is controlled by time as well as number of cleavage divisions, 2) that day 4 embryos are more likely to have completed more cleavage divisions than day 3 embryos, 3) that day 4 embryos represent retarded embryos more susceptible to apoptosis.

Absence of heat-induced apoptosis in embryos heat shocked at the 2- and 4-cell stage is associated with a lack of activation of group II caspases including caspase 3. Failure of activation of these execution caspases is probably not a reflection of the absence of these enzymes or the absence of much of the signaling pathway for caspase activation. Experiments using the protein kinase inhibitor staurosporine indicates that the cell death machinery is constitutively present in early cleavage embryos. Staurosporine could induce apoptosis in mouse embryos at the 1-4 cell stage (Well et al., 1996; Exley et al., 1999) and bovine embryos at the 1-16 cell stage (Matwee et al., 2000). Also mRNA for caspases-2, -3, -6 and -12 are present throughout preimplantation embryonic development in the mouse as are transcripts for the antiapoptotic Bcl-2, Bcl-xl and Bcl-w and the proapoptotic Bax (Exley et al., 1999). However, certain aspects of cell death are altered in early embryos since the time required for staurosporine to induce apoptosis in zygotes and 1-4 cell stage embryos was longer than for blastocysts or other cell types (Well et al., 1996). One possibility is that anti-apoptotic proteins are very high in early embryos and that heat-induced apoptosis becomes possible when amounts of these proteins decline during development.

It is also possible that the particular mechanisms for heat-induced apoptosis are dysfunctional in early cleavage-stage embryos. For heat shock and oxidative stress, induction of apoptosis is initiated upon activation of the enzyme sphingomyelinase which hydrolyzes the membrane phospholipid sphingomyelin to generate the second messenger ceramide (Verheij et al., 1996; Haimovitz-Friedman et al., 1997; Pena et al., 1997). Perhaps, amounts of this enzyme or its substrate early in development make the early embryo unable to generate sufficient ceramide in response to heat shock for apoptosis to occur. Finally, the fact that heat-induced apoptosis first occurs at a stage of development coincident with embryonic genome activation in the cow (Memili and First, 2000) means that most likely heat-induced apoptosis is dependent on transcriptionally-controlled events in the embryo.

Previous exposure of day 4 embryos at the 8-16 cell stage to a mild and transient heat shock of 40°C for 80 min blocked the induction of apoptosis induced by a subsequent severe heat shock of 41°C for 9 h. Such a phenomenon has been seen for others cells also (Boreham et al., 1997; Buzzard et al., 1998; McMillan et al., 1998). The biochemical mechanisms by which mild heat shock prevents apoptosis induced by a more severe heat shock presumably involves HSP70 (Mosser and Martin, 1992; Mosser et al., 1997; Li et al., 2000a; Mosser et al., 2000; Saleh et al., 2000; Beere and Green, 2001). Besides protecting cells from heat shock, HSP70 can protect cells against several apoptotic stimuli including DNA damage, UV irradiation, serum withdrawal, and chemotherapeutic agents (Samali and Orrenius, 1998). The mechanism by which HSP70 exerts its anti-apoptotic action is not completely understood but it can block multiple points along the apoptotic pathway. Heat shock protein 70 and 72 inhibits poly-(ADP-ribose) polymerase cleavage (Mosser et al., 1997; Buzzard et al., 1998). Heat shock protein 70 also blocks cytochrome c release from mitochondria (Mosser et al., 2000), processing of inactive procaspase-3 (Mosser et al., 1997), and SAPK/JNK activation (Mosser et al., 1997). In addition, disruption of murine heat shock factor 1 gene increased heat-induced apoptosis (McMillan et al., 1998). The bovine embryo can produce increased amount of HSP70 in response to heat shock as early as the 2-cell stage (Edwards and Hansen, 1996; Edwards et al., 1997; Chandolia et al., 1999).

Heat shock also compromised embryonic viability as determined by the total number of cells per embryo at 24 h after the initiation of heat shock. The deleterious effects of heat shock on embryo cell number depended upon the magnitude of heat shock and stage of embryonic development. Thus, heat shock at the 2-4 cell stage caused a larger reduction in embryo cell number than heat shock at the morula stage. This finding is in agreement with earlier studies in which heat shock caused a greater reduction in development when applied at the two cell stage than the morula stage (Aréchiga et al., 1995; Edwards and Hansen, 1997) or at day 3 after fertilization than at day 4 (Ju et al., 1999). Therefore, those embryos that were at stages of development capable of heat-induced apoptosis were more resistant to the deleterious effects of heat shock on development. One possibility is that the sensitivity of the early embryo to heat shock is a reflection, at least in part, of the failure of the embryos to undergo heat-induced apoptosis to remove damaged cells from the embryonic lineage. There is some evidence that induction of limited apoptosis by heat shock does not necessarily inhibit embryonic development since embryos ≥ 16 cells that experienced apoptosis induced by 40 or 41°C did not have reduced cell number 24 h after heat shock. Further studies evaluating other indices of development as well as the effects of inhibitors of apoptosis on development should add insight into the implications of stress-induced apoptosis for embryonic survival.

CHAPTER 4
APOPTOSIS IS AN ADAPTIVE RESPONSE IN BOVINE PREIMPLANTATION EMBRYOS THAT FACILITATES SURVIVAL AFTER HEAT SHOCK

Introduction

Apoptosis is a highly conserved form of cell death that plays a major role in animal development and cellular homeostasis by acting as a quality control mechanism to remove cells that are damaged, nonfunctional, misplaced or supranumerary (Jacobson et al., 1997). Cellular changes associated with death caused by apoptosis typically lead to the removal of cells without induction of inflammation that might otherwise compromise integrity of the surrounding cells or tissues (Meier et al., 2000). Use of the TUNEL assay to detect oligonucleososmal DNA fragments has revealed the presence of apoptotic cells in preimplantation embryos from several species (Brison and Schultz, 1997; Byrne et al., 1999; Hardy, 1999; Matwee et al., 2000). Spontaneous apoptosis is first observed in bovine embryos at the 8-16 cell stage (Byrne et al., 1999; Matwee et al., 2000) at a time coincident with major activation of embryonic genome (Memili and First, 2000).

Exposure of preimplantation embryos to an adverse environment can increase the number of apoptotic cells. Both heat shock (Chapter 3) and arsenic (Krininger III et al., 2002), for example, increase the proportion of TUNEL-positive blastomeres and caspase activity in bovine embryos. The induction of apoptosis by heat shock in cattle embryos is developmentally regulated and does not occur until day 4 after insemination (Chapter 3). This pattern of development parallels embryonic resistance to heat shock since embryos acquire increased resistance to heat shock (as determined by ability to continue development following heat shock) as they progress from the 2-cell stage to the morula stage (Edwards and Hansen, 1997). Such a correlation suggests that apoptosis may be one of the mechanisms preimplantation bovine embryos acquire to allow survival after heat shock. Given that heat shock only induces apoptosis in a fraction of cells (15-29%), and not the majority of blastomeres, apoptosis may ensure that damaged cells are removed from the heat shocked embryo so as to facilitate further development.

For the current studies, it was hypothesized that heat-induced apoptosis in bovine embryos is mediated by group II caspases and that the removal of heat-damaged cells by apoptosis is beneficial to embryonic development. Experiments were performed to 1) test whether the group II caspase inhibitor z-DEVD-fmk decreases caspase activity in bovine embryos exposed to heat shock of 41°C; 2) test whether z-DEVD-fmk decreases TUNEL-positive cells in bovine embryos exposed to heat shock of 41°C; 3) and evaluate whether blocking apoptosis with z-DEVD-fmk decreases development to the blastocyst stage in bovine embryos exposed to heat shock of 41°C .

Materials and Methods

Materials

Materials for in vitro maturation, fertilization and culture were purchased as described in Chapter 3. Modified Tyrode's solutions were obtained from Cell and Molecular Technologies (Lavallette, NJ) to prepare HEPES-TALP, IVF-TALP and Sp-TALP (Parrish et al., 1986). Potassium Simplex Optimized Medium was obtained from Cell and Molecular Technologies. The KSOM, which contains 1 mg/ml BSA, was modified on the day of use by adding an additional 2 mg/ml EFAF-BSA, 2.5 µg/ml gentamicin, essential amino acids (Basal Medium Eagle) and non-essential amino acids

(Minimum Essential Medium) purchased from Sigma Chemical Company (St. Louis, MO). Oocyte collection medium was TCM-199 with Hank's salts without phenol red and supplemented with 2% (v/v) bovine steer serum (containing 2 U/ml heparin), 100 U/ml penicillin-G, 0.1 mg/ml streptomycin, and 1 mM glutamine. Oocyte maturation medium was TCM-199 with Earle's salts supplemented with 10% (v/v) steer serum, 22 μg/ml sodium pyruvate, 20 μg/ml FSH, 2 μg/ml estradiol 17-β, 50 μg/ml gentamicin and an additional 1 mM glutamine. Other materials for in vitro fertilization were purchased as described in Chapter 3.

In Situ Cell Death Detection Kit (fluorescein) and propidium iodide were from Roche Diagnostics Corporation (Indianapolis, IN) and Sigma Chemical Company (St. Louis, MO)., respectively. Polyvinylpyrrolidone was purchased from Eastman Kodak Company (Rochester, NY). Prolong Antifade Kit was obtained from Molecular Probes (Eugene, Oregon), RQ1 RNA-free DNase from Promega (Madison, WI), RNase A from Qiagen (Valencia, CA), PhiPhiLux-G_1D_2 from OncoImmunin, Inc. (Gaithersburg, MD), and z-DEVD-fmk (FMK004) was from R&D systems (Minneapolis, MN). Other reagents were purchased from Fisher (Pittsburgh, PA) or Sigma Chemical Company (St. Louis, MO).

In Vitro Production of Embryos

Procedures were as described earlier in Chapter 3. Briefly, COCs were obtained by slicing 2-10 mm follicles on the surface of the ovary into a beaker containing oocyte collection medium. Cumulus-oocyte complexes which had at least one layer of compact cumulus cells were washed two times and matured in groups of 10 in 50 μl drops of oocyte maturation medium overlaid with mineral oil for 22 h at 38.5°C in an atmosphere of 5% (v/v) CO_2 in humidified air. For fertilization, groups of 30 COCs were transferred to four well plates containing 600 µl IVF-TALP per well. Oocytes were fertilized with 25 µl sperm suspension and 25 µl PHE [0.5 mM penicillamine, 0.25 mM hypotaurine and 25 µM epinephrine in 0.9% (w/v) NaCl] added to each well. Presumptive zygotes were removed from fertilization wells, denuded of cumulus cells, washed 2-3 times in HEPES-TALP to remove remaining cumulus cells and associated spermatozoa and placed in groups of 25-30 in 50 µl drops of modified KSOM overlaid with mineral oil at 38.5°C and 5% CO_2 (v/v) in humidified air. Embryos $\geq$ 16 cell stage were harvested from culture at day 4 after insemination and subjected to treatments.

TUNEL and Propidium Iodide Labeling

Embryos were subjected to TUNEL analysis using one of two procedures - after embryos were fixed to microscope slides or while embryos were in solution. The procedure for TUNEL analysis on embryos affixed to slides was described previously in Chapter 3. For embryos in solution, the following procedure was performed. Embryos were washed once in PBS-PVP and permeabilized in 4-well plates containing 500 µl 0.5% (v/v) Triton X-100 containing 0.1% (w/v) sodium citrate for 1 h at room temperature. Positive controls were incubated in 50 µl drop RQ1 RNase-free DNase (50 U/ml) at 37°C for 1 h. Embryos were washed in PBS-PVP and incubated with 50 µl drop of TUNEL reaction mixture (containing fluorescein isothiocyanate-conjugated dUTP and the enzyme terminal deoxynucleotidyl transferase), as prepared by the manufacturer, for 1 h at 37°C in the dark. Negative controls were incubated in the absence of terminal deoxynucleotidyl transferase. Embryos were then incubated in 4-well plates containing 500 µl RNase A (50 µg/ml) for 1 h at room temperature, followed by 500 µl 0.5 µg/ml of propidium iodide for 30 min at room temperature. Embryos were washed 3 times in PBS-PVP to remove excess propidium iodide, placed on micrsocope slides and mounted with 16 µl mounting medium containing Antifade as recommended by the manufacturer. TUNEL labeling was observed using a Zeiss Axioplan 2 fluorescence microscope with dual filter. Each embryo was analyzed for total number of nuclei and number of TUNEL-labeled nuclei.

Caspase Activity

To measure caspase activity, embryos were removed from culture medium and washed 3 times in 50 µl drops of prewarmed HEPES-TALP. Embryos were incubated in 25 µl microdrops of HEPES-TALP containing 5 µM PhiPhiLux-G_1D_2 at 39°C for 40 min in the dark. Negative controls were incubated in HEPES-TALP only. Following incubation, embryos were washed twice in 50 µl drops of HEPES-TALP, placed on poly-l-lysine coated slides, and mounted with a coverslip. Caspase activity was determined immediately after the end of heat shock using a Zeiss Axioplan 2 fluorescence microscope with a X 45 objective. Images were obtained using a Spot camera and software (Diagnostic Instruments, Inc.). Pictures were taken using a background subtract feature from the spot software and digital images were stored as .tiff files. Fluorescence intensity was quantified using IPLab for MacIntosh version 3.5 (BioVision Technologies, Inc., Exton, PA). Each embryo represented a region of interest (ROI). A circular draw was manually performed for each ROI and the pixel intensity per unit area was determined.

Effect of z-DEVD-fmk on Heat-Induced Caspase Activity in Bovine Embryos

The experiment was designed as a 2 x 2 factorial arrangement of treatments to determine whether z-DEVD-fmk blocks heat-induced caspase activity. Embryos ≥ 16-cell stage were collected on day 4 after insemination and transferred to a new drop of modified KSOM containing 200 µm z-DEVD-fmk reconstituted in 1% (v/v) DMSO or 1% (v/v) DMSO alone (vehicle) at 38.5°C for 15 h. After this preincubation period, embryos were either maintained at 38.5°C or exposed to 41°C for 9 h. At the end of heat shock, embryos were washed 3 times in 50 µl drops of prewarmed HEPES-TALP and then incubated in 25 µl drop HEPES-TALP containing 5 µM PhiPhiLux-G_1D_2 at 39°C for 40 min in the dark. Caspase activity was then determined. The experiment was replicated 4 times using 57-77 embryos/treatment.

Effect of z-DEVD-fmk on Heat-Induced Changes in TUNEL-Positive Blastomeres and Embryo Cell Number

Embryos ≥ 16-cell stage were collected on day 4 after insemination and transferred to a new drop of modified KSOM containing 200 µm z-DEVD-fmk reconstituted in 1% (v/v) DMSO or 1% (v/v) DMSO alone (vehicle) at 38.5°C for 15 h. After preincubation, embryos were either maintained at 38.5°C for 24 h or exposed to 41°C for 9 h and 38.5°C for 15 h. Embryos were then processed for analysis by TUNEL. The experiment was replicated 2 times using 27-37 embryos/treatment.

Effect of z-DEVD-fmk on Development of Embryos After Heat Shock

Embryos ≥ 16-cell stage were collected on day 4 after insemination and transferred to a new drop of modified KSOM containing 200 µm z-DEVD-fmk reconstituted in 0.5-1% (v/v) DMSO or 0.5-1% (v/v) DMSO alone (vehicle) at 38.5°C for 15 h. After preincubation, embryos were either maintained at 38.5°C continuously or exposed to 41°C for 6-9 h followed by culture at 38.5°C continuously and the percentage of embryos developing to the blastocyst stage was determined on day 8 after insemination. The experiment was replicated 15 times using 315-324 embryos/group.

Statistical Analysis

Data were analyzed by least-squares analysis of variance. Independent variables were temperature, treatment and replicate. The mathematical model included main effects and all interactions. Two analyses were performed: by the GLM procedure of SAS (SAS, 1989), where replicate (i.e., day of IVF procedure) was considered a fixed variable, and by the Mixed Models procedure of SAS, where replicate was considered a random variable. Probability values were similar for both methods and probability values reported herein are derived from the analysis by GLM. Percentage data were analyzed without transformation and after being subjected to the arcsin transformation. Unless stated otherwise, probability values were similar for both analyses; least-squares means reported here are derived from the analysis of untransformed data while probability levels are derived from the analysis of transformed data.

Results

Effect of z-DEVD-fmk on Heat-Induced Caspase Activity in Bovine Embryos

Representative digital images illustrating caspase activity in embryos ≥ 16 cell stage are shown in Figure 4-1. For control embryos, heat shock of 41°C for 9 h increased caspase activity (Figure 4-1A-B). However, heat shock did not increase caspase activity in embryos cultured in z-DEVD-fmk (Figure 4-1C-D). Caspase activity was quantified and subjected to analysis of variance (Figure 4-2). There was a treatment x temperature interaction ($p < 0.01$) affecting caspase activity. Exposure of control embryos to 41°C for

9 h increased caspase activity as compared to 38.5°C. However, culturing embryos in the presence of z-DEVD-fmk reduced caspase activity caused by heat shock of 41°C for 9 h.

Effect of z-DEVD-fmk on Heat-Induced Changes in TUNEL-Positive Blastomeres and Embryo Cell Number

Heat shock of 41°C for 9 h increased the percentage of TUNEL-positive cells in control embryos. However, there was no increase in the percentage of TUNEL-positive cells after heat shock in embryos cultured in z-DEVD-fmk (treatment x temperature, $p< 0.05$ for untransformed data; Figure 4-3A). Moreover, heat shock of 41°C for 9 h reduced ($p< 0.001$) total cell number in control (vehicle) or z-DEVD-fmk embryos. There was no effect of z-DEVD-fmk on total cell number (Figure 4-3B).

Effect of z-DEVD-fmk on Development of Embryos After Heat Shock

Heat shock of 41°C for 9 h reduced the percentage of embryos developing to the blastocyst stage for both control (vehicle) and z-DEVD-fmk embryos. However, the decrease in development was greater for z-DEVD-fmk embryos (treatment x temperature, $p< 0.05$; Figure 4-4).

Discussion

During the course of preimplantation development, disruption in the microenvironment of the embryo can lead to inhibition of development and embryonic death. For at least two embryonic stresses, heat shock (Chapter 3) and arsenic (Krininger III et al., 2002), affected embryos undergo an increase in the proportion of cells that are labeled with the TUNEL reaction. While the presence of TUNEL-positive cells is usually indicative of apoptosis, necrosis and artefacts caused by sample fixation can also lead to TUNEL labeling (Hardy, 1999). The present study provides strong evidence that the increase in TUNEL-positive cells in bovine preimplantation embryos caused by heat

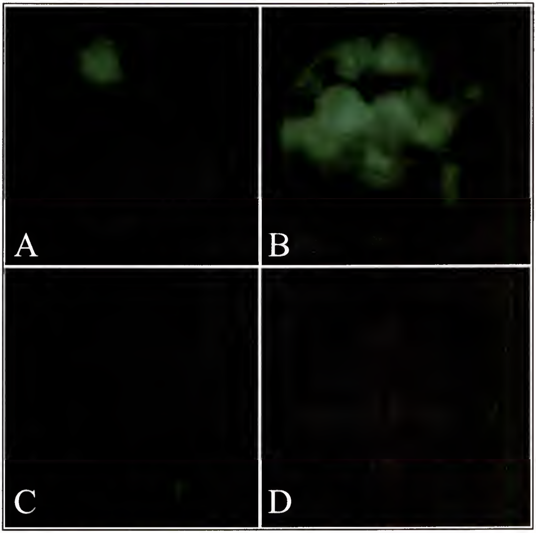

Figure 4-1. Representative digital images illustrating group II caspase activity in embryos $\geq$ 16 cell stage. Panels A (38.5°C) and B (41°C for 9 h) are control embryos cultured in medium containing the DMSO vehicle. Panels C (38.5°C) and D (41°C for 9 h) are embryos cultured in z-DEVD-fmk. Note that exposure of control embryos to heat shock of 41°C for 9 h increased caspase activity. This increase did not occur for embryos cultured in z-DEVD-fmk.

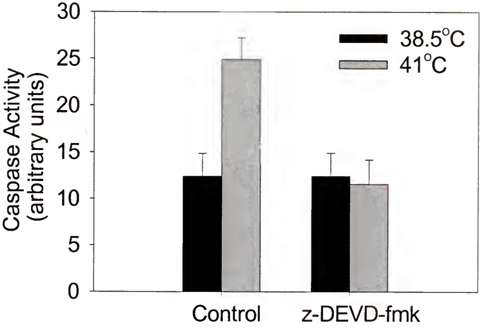

Figure 4-2. Effect of z-DEVD-fmk on heat-induced caspase activity in bovine embryos. Results are least-squares means ± SEM of 4 replicates using 57-77 embryos/treatment. Exposure of control embryos to 41°C for 9 h increased caspase activity as compared to 38.5°C. Culture of embryos in the presence of z-DEVD-fmk, however, blocked the increase in caspase activity caused by heat shock (treatment x temperature, $p < 0.01$).

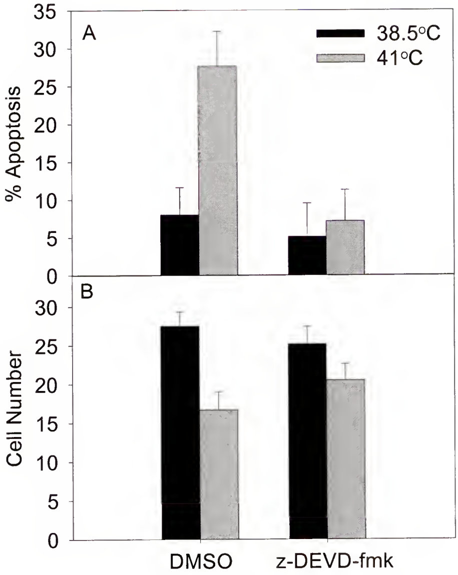

Figure 4-3. Effect of z-DEVD-fmk on heat-induced changes in TUNEL-positive blastomeres and embryo cell number. Results are least-squares means ± SEM of 2 replicates using 27-37 embryos/treatment. For control embryos, heat shock of 41°C for 9 h increased the percentage of TUNEL-positive cells. For embryos cultured in z-DEVD-fmk, however, there was no increase in the percentage of TUNEL-positive cells after heat shock (treatment x temperature, $p < 0.05$ for untransformed data). Heat shock of 41°C for 9 h reduced ($p < 0.001$) embryo total cell number but there was no treatment x temperature interaction.

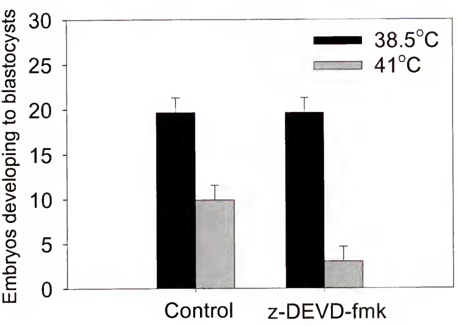

Figure 4-4. Effect of z-DEVD-fmk on development of embryos after heat shock. Results are least-squares means ± SEM of 15 replicates using 315-324 embryos/group. Heat shock of 41°C for 9 h reduced development of control embryos to the blastocyst stage. Moreover, the deleterious effects of heat shock on development was exacerbated by culturing embryos in z-DEVD-fmk (treatment x temperature, $p < 0.05$).

shock is in fact indicative of apoptosis. This is so because heat shock caused an increase in group II caspase activity and because preincubation of embryos in z-DEVD-fmk blocked heat-induced caspase activity and prevented the increase in TUNEL-positive blastomeres observed after heat shock. Thus, activation of the apoptotic machinery is one consequence of embryonic exposure to heat shock. Moreover, results indicate that heat-shock induced apoptosis represents an adaptive, thermotolerizing response in preimplantation bovine embryos because inhibition of the capacity for apoptosis with z-DEVD-fmk compromised ability of embryos to continue development after heat shock.

This is the first report to indicate a beneficial role for apoptosis in allowing the preimplanation embryo to overcome stress. While others have reported apoptosis in embryos (Brison and Schultz, 1997; Byrne et al., 1999; Hardy, 1999; Matwee et al., 2000), it is generally assumed that apoptosis represents an embryo at risk. Indeed, this may be true and certain treatments which increase development also decrease apoptosis (Brison and Schultz, 1997; Kolle et al., 2002; Makarevich and Markkula, 2002). However, it is simplistic to view apoptosis as solely a marker of embryo stress without realizing that the process itself can contribute to the survival of the embryo in response to stress. Cell loss itself does not inexorably lead to embryonic death, and numerous experiments have demonstrated the production of offspring does result from hemi-sectioning the embryos to produce identical twins (see Martinod et al., 1991 for example). The mechanism by which inhibition of apoptosis reduces development in heat-shocked embryos remains unknown. One possibility is that cells that are not removed by apoptosis subsequently undergo death more typical of necrosis (Xiang et al., 1996; Woo et al., 1998). Also, apoptosis may prevent a damaged cell from contributing abnormal daughter cells to the embryo.

Given that apoptosis represents a mechanism to limit effects of heat shock on embryos, it is likely that the capacity of an embryo for apoptotic responses determines, at least in part, its resistance to heat shock. Heat shock cannot induce apoptosis in embryos less than the 8-cell stage as demonstrated in Chapter 3, and embryos before this time experience a greater inhibition of development following heat shock than do embryos after the 8-cell stage (Edwards and Hansen, 1997). Thus, one reason that early bovine embryos are more sensitive to heat shock than preimplantation embryos at later stages of development may be the inability of early embryos to undergo heat-induced apoptosis.

One of the features of heat-induced apoptosis in preimplantation bovine embryos is that only a limited proportion of cells undergo apoptosis following heat shock. In the present study, for example, 27.6% of blastomeres were positive for TUNEL labeling after exposure to 41°C for 9 h. It is possible that more extensive apoptosis, such as might occur following more severe stress, would compromise embryonic development. Thus, the degree of the apoptotic response may be an important determinant in embryonic fate. While limited apoptosis is a mechanism for embryonic homeokinesis to remove damaged cells without disturbing the embryonic potential to develop further, more extensive apoptosis may leave the embryo with too few cells to sustain development. Given the importance of apoptosis in determining embryonic survival in response to stress, manipulation of apoptosis responses in embryos may lead to new methods for improving systems for in vitro production of embryos or for reducing embryonic mortality in female animals.

CHAPTER 5

GENETIC DIVERGENCE IN CELLULAR RESISTANCE TO HEAT SHOCK IN CATTLE: DIFFERENCES BETWEEN BREEDS DEVELOPED IN TEMPERATE VS HOT CLIMATES IN RESPONSES OF PREIMPLANTATION EMBRYOS, REPRODUCTIVE TRACT TISSUES AND LYMPHOCYTES TO ELEVATED CULTURE TEMPERATURES

Introduction

Mammalian species have evolved to be adapted for different ecosystems. Among other characteristics, domestic cattle can be distinguished on the basis of adaptation for life in hot climates. Breeds of cattle of *Bos indicus* origin such as Brahman as well as some *B. taurus* breeds (for example, Senepol) are more resistant to tropical conditions such as elevated temperature and humidity than breeds that evolved in Europe such as Angus and Holstein. Most of this adaptation to elevated temperature is due to superior ability of thermotolerant breeds to regulate body temperature (Adeyemo et al., 1979; Bennett et al., 1985; Hammond et al., 1996; Gaughan et al., 1999). There are also indications that breed differences in thermal resistance extend to the cellular level. For example, DNA synthesis by cultured endometrium was reduced by heat shock to a greater degree for Holstein than Brahman (Malayer and Hansen, 1990a). Additionally, the decrease in lymphocyte viability caused by heat shock of 42 or 45°C was greater for lymphocytes from Angus cows as compared to lymphocytes collected from Brahman or Senepol cattle (Kamwanja et al., 1994).

Failure of cows to maintain homeothermy when exposed to elevated ambient temperature can compromise fertility. Heat-stress induced infertility is a multifactorial problem since hyperthermia can affect cellular function in different tissues of the female reproductive tract (Wolfenson et al., 2000; Hansen et al., 2001). Embryos in particular are very susceptible to elevated temperature. Exposure of cows to heat stress during early pregnancy reduces embryonic survival (Ealy et al., 1993) and culture of embryos at elevated temperatures reduces development to the blastocyst stage (Edwards and Hansen, 1997; Rivera and Hansen, 2001; Al-Katanani and Hansen, 2002).

The hypothesis of this experiment was that cells and tissues from Brahman and Senepol cattle (thermotolerant breeds) are better able to survive and function after exposure to elevated temperature than cells and tissues from Holstein and Angus cattle (thermosensitive breeds). Analyses were performed to determine 1) ability of embryos to develop to the blastocyst stage after exposure to elevated temperature, 2) the magnitude of heat-shock induced changes in protein secretion by cultured endometrium and oviduct, 3) apoptosis responses in lymphocytes, and 4) depletion of the antioxidant GSH in lymphocytes. Apoptosis responses were evaluated because heat shock can induce apoptosis in many cell types (Mosser et al., 1997; Sodja et al., 1998). Glutathione was evaluated because it is an antioxidant that has been implicated in preventing the deleterious effects of heat hock in preimplantation embryos (Aréchiga et al., 1995). Thus, we used lymphocytes as a model to test whether genetic control of cellular resistance to heat shock involved differences in apoptosis or antioxidant status. The temperature used to evaluate heat shock effects was 41°C. Such a temperature is near the high range of rectal temperatures of lactating cows in Florida exposed to heat stress (Rivera and Hansen, 2001).

Materials and Methods

Materials

Materials for in vitro maturation, fertilization and culture were purchased as described in Chapter 3. Oocyte collection medium was TCM-199 with Hank's salts without phenol red and supplemented with 2% (v/v) bovine steer serum (containing 2 U/ml heparin), 100 U/ml penicillin-G, 0.1 mg/ml streptomycin, and 1 mM glutamine. Oocyte maturation medium was TCM-199 with Earle's salts supplemented with 10% (v/v) steer serum, 22 μg/ml sodium pyruvate, 20 μg/ml FSH, 2 μg/ml estradiol 17-β, 50 μg/ml gentamicin and an additional 1 mM glutamine. Frozen semen from various bulls was purchased from American Breeders Service (Madison, WI) or were from a repository at USDA-ARS Subtropical Agricultural Research Station (Brooksville, FL). Modified Tyrode's solutions were obtained from Cell and Molecular Technologies (Lavallette, NJ) to prepare HEPES-TALP, IVF-TALP and Sp-TALP (Parrish et al., 1986). Potassium Simplex Optimized Medium was obtained from Cell and Molecular Technologies. The KSOM, which contains 1 mg/ml BSA, was modified on the day of use by adding an additional 2 mg/ml essentially fatty acid free BSA, 2.5 μg/ml gentamicin, essential amino acids (Basal Medium Eagle) and non-essential amino acids (Minimum Essential Medium) purchased from Sigma Chemical Company (St. Louis, MO).

Eagle's Minimum Essential Medium (MEM) was purchased from Sigma Chemical Company (St. Louis, MO). Pig MEM (a modification of MEM) was prepared as previously described (Malayer et al., 1988). L-[4,5-^{3}H]leucine (specific activity: 173 Ci/mmol) was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Prostaglandin $F_{2\alpha}$ (Lutalyse®) was from Pharmacia & Upjohn (Kalamazoo, MI) and

GnRH (Cystorelin) was from Sanofi Animal Health (Overland Park, Kansas). The medium for lymphocyte culture was TCM-199 supplemented with 5% (v/v) horse serum, 200 U/ml penicillin-G, 0.2 mg/ml streptomycin, 1 mmol l^{-1} extra glutamine and 1 x 10^{-5} mol l^{-1} β-mercaptoethanol. Glutathione reductase, β-nicotinamide adenine dinucleotide phospahate reduced form (NADPH) and 5,5-dithiobis(2-nitrobenzoic acid) (DNTB) were from Sigma Chemical Company (St. Louis, MO). In Situ Cell Death Detection Kit (fluorescein) and propidium iodide were obtained from Roche Diagnostics Corporation (Indianapolis, IN) and Sigma Chemical Company (St. Louis, MO), respectively. Polyvinylpyrrolidone was purchased from Eastman Kodak Company (Rochester, NY). Prolong Antifade® Kit was obtained from Molecular Probes (Eugene, Oregon), RQ1 RNA-free DNase was from Promega (Madison, WI) and RNase A was from Qiagen (Valencia, CA). Fico/Lite LymphoH® was obtained from Atlanta Biologicals (Norcross, GA) and red blood cell lysis buffer was from Sigma Chemical Company (St. Louis, MO). Other reagents were purchased from Fisher (Pittsburgh, PA) or Sigma Chemical Company (St. Louis, MO).

Animals

The experiment was performed using non-lactating cows: 20 Brahman, 11 Senepol, 20 Holstein and 20 Angus. Cows were moved to a common facility at the USDA-ARS and housed together for 20 weeks to ensure they had been exposed to similar environmental conditions. After this period, the experiment was conducted during March-June, 2000. For each of 10 replicates (one replicate per week), estrous cycles of 8 cows (2 per breed) were synchronized with the administration of GnRH (100 µg, i.m.) on day 0, $PGF_{2\alpha}$ (12.5 mg, i.m.) on day 7, $PGF_{2\alpha}$ (12.5 mg, i.m.) on day 8 and GnRH (100 μg, i.m.) on day 9. A 40-ml blood sample was collected from the jugular vein on day 0 (day of first GnRH injection) and transported to the laboratory. Cows were slaughtered on day 12 after the first GnRH injection (~ day 2 after estrus). Ovaries were obtained after slaughter and morphological structures on each ovary were recorded (corpus luteum, corpus hemorraghicum, corpus albicans and large preovulatory follicle) to determine whether ovulation occurred.

In Vitro Production of Embryos

After slaughter and examination for ovarian structures, ovaries were sorted by breed, placed into buckets containing sterile saline [0.9% (w/v) NaCl solution] supplemented with 100 units/ml penicillin-G and 0.1 mg/ml streptomycin and transported to the laboratory at ambient temperature. Ovaries were washed several times with prewarmed sterile saline at 25-30°C to remove blood and debris. For each replicate, COCs were pooled from a pair of cows from each breed. Cumulus-oocyte complexes were obtained by slicing 2-10 mm follicles on the surface of the ovary into a beaker containing oocyte collection medium. Cumulus-oocyte complexes which had at least one layer of compact cumulus cells were washed two times and matured in groups of 10 in 50 μl drops of oocyte maturation medium overlaid with mineral oil for 22 h at 38.5°C in an atmosphere of 5% (v/v) CO_2 in humidified air. For fertilization, groups of 30 COCs were transferred to four well plates containing 600 μl IVF-TALP per well. Frozen-thawed sperm from each breed (3 straws; a separate bull was used for each replicate to avoid confounding breed effects with bull) were purified by centrifugation on a Percoll gradient and resuspended in IVF-TALP. Oocytes were fertilized with semen from the same breed by adding 25 μl sperm suspension to give an approximate concentration of 1×10^6

spermatozoa per well and 25 µl PHE [0.5 mM penicillamine, 0.25 mM hypotaurine and 25 µM epinephrine in 0.9% (w/v) NaCl] to each well. Presumptive zygotes were removed from fertilization wells, denuded of cumulus cells, washed 2-3 times in HEPES-TALP to remove remaining cumulus cells and associated spermatozoa and placed in groups of 15-20 presumptive zygotes in 50 µl drops of modified KSOM overlaid with mineral oil at 38.5°C and 5% CO_2 (v/v) in humidified air.

At day 4 after insemination, stage of embryonic development and cleavage rate was determined. Embryos > 8 cell stage were harvested from culture and randomly distributed to 25 µl drops of modified KSOM at either 38.5°C continuously or were exposed to heat shock of 41°C (7% CO_2) for 6 h followed by culture at 38.5°C (5 % CO_2) thereafter. Embryos were examined at this stage since this represents a period when the embryonic genome was activated and breed differences would be likely. The duration of heat shock was chosen to reduce development while avoiding exposing embryos to conditions severe enough to obscure breed differences in resistance to heat shock. Moreover, the period of 6 h is within the range of time that mean temperatures of heat-stressed, lactating cows in Florida experienced rectal temperatures above 40°C (1-8.5 h depending upon coat color; Rivera and Hansen, 2001). The number of embryos per 25 µl drop of modified KSOM was fixed within runs and ranged between 5-10. The proportion of embryos that developed to the blastocyst stage was recorded at day 8 and 9 after insemination. Day 9 embryos were then processed for determination of total cell number using propidium iodide staining.

For statistical analysis, oocyte recovery rate, cleavage rates and the percent embryos developing to the blastocyst stage were calculated for each replicate [i.e., pair of cows within a breed; n=10 for all breeds except Senepol where n=6 (5 pairs and 1 single cow]. The total number of oocytes examined for cleavage rate was 466 (Angus), 432 (Holstein), 784 (Brahman) and 295 (Senepol). The number of embryos that were cultured was 115 (Angus-38.5°C), 115 (Angus-41° C), 111 (Holstein-38.5°C), 127 (Holstein-41°C), 258 (Brahman-38.5°C), 256 (Brahman-41°C), 89 (Senepol-38.5°C) and 83 (Senepol-41°C).

Determination of Embryo Cell Number

Embryos were washed 2 times in 70 μl drops of PBS [10 mmol l^{-1} potassium phosphate, 0.9 % (w/v) NaCl, pH 7.4] containing 1 mg/ml polyvinylpyrrolidone (PBS-PVP) by transferring the embryos from drop to drop. Zona pellucida-intact embryos were fixed in 70 μl drops of 4% (w/v) paraformaldehyde in 100 μmol l^{-1} phosphate with 0.9 % (w/v) NaCl for 1 h at room temperature, washed twice in PBS-PVP, transferred to a poly-l-lysine coated slide and allowed to dry for 24 h at room temperature. Embryos were washed twice in PBS-PVP, incubated with RNase A (50 μg/ml) for 1 h at room temperature, and then with 0.5 μg/ml propidium iodide for 30 min at room temperature. Embryos were washed 4 times in PBS-PVP to remove excess propidium iodide and mounted with coverslips with 16 μl mounting medium containing Prolong Antifade®. Propidium iodide labeling was observed using a Zeiss Axioplan 2 fluorescence microscope with Texas red filter. The number of embryos analyzed for cell number was 84 (Angus-38.5°C), 62 (Angus-41°C), 53 (Holstein-38.5°C), 70 (Holstein-41°C), 133 (Brahman-38.5°C), 139 (Brahman-41°C), 61 (Senepol-38.5°C) and 40 (Senepol-41°C).

Oviductal and Endometrial Tissue

For a subset of cows, oviducts (from 14 Angus, 14 Holstein, 15 Brahman, and 10 Senepol cows) and uteri (from12 Angus, 12 Holstein, 13 Brahman and 7 Senepol cows) were obtained after exsanguination and transported to the laboratory at ambient temperature. The oviducts were trimmed free of connective/vascular tissue and wiped with 70 % (v/v) ethanol to remove blood and debris. Explants of endosalphinx were collected by stripping the oviducts of each cow. The oviducts were squeezed from the isthmus end using sterile forceps and tissue from both oviducts of a single cow were pooled together. Explants from each cow were divided into 2 equal amounts of tissue and placed in 60 mm petri dishes containing 5 ml of Pig MEM with 0.1 times the normal leucine concentration. The tissue wet weight was recorded (~ 250 mg/dish). Tissue samples of intercaruncular endometrium from both uterine horns were collected, cut into 2-5 mm^3 explants, washed 2-3 times, divided into four 100 mm petri dishes containing 15 ml Pig MEM with 0.1 times the normal leucine concentration, and the tissue wet weight was recorded (~ 500 mg/dish). Oviductal and endometrial cultures were supplemented with 10 μCi [^{3}H]leucine. Oviductal explants were cultured at 38.5°C or 41°C for 9 h. Endometrial explants were cultured at 38.5°C or 41°C for 3 or 9 h. Oviduct and endometrium was exposed to heat shock of a longer duration than for embryos because of the assumption that embryonic development is more sensitive to heat shock than protein secretion by endometrium or oviduct; the goal was to apply a heat shock great enough to compromise tissue function so breed x temperature interactions could be tested. At the end of the culture period, medium was harvested, centrifuged (1000 x g; 10 min; 4°C) and stored at - 20°C. Incorporation of [^{3}H]leucine into newly synthesized protein secreted into the medium was determined by trichloroacetic acid (TCA) precipitation.

Trichloroacetic Acid Precipitation

For this procedure, one inch square pieces of filter paper were soaked in 20% (w/v) TCA and allowed to dry. Aliquots (50 µl) of endometrial or oviductal medium from explants cultures were pipetted in duplicate on the filter paper and air dried. Filter papers were then sequentially soaked in 20% (w/v) TCA for 10 min, 5% (w/v) TCA for 20 min, 5 % (w/v) TCA for 10 min and 95% (v/v) ethanol for 10 min, and 95% (v/v) ethanol for 5 min. Filter papers were then dried and subjected to scintillation spectrometry.

Preparation of Lymphocytes

Lymphocytes were cultured as previously described (Kamwanja et al., 1994) except that the medium used was chosen because it was optimal for support of lymphocyte proliferation in sheep (Gottshall and Hansen, 1994) and also supported mitogen-induced lymphocyte proliferation in cattle (unpublished results). Jugular venous blood (40 ml) was centrifuged at 2000 x g for 30 min. The buffy coat was removed and mixed into 2 ml of TCM-199. The cell suspension was transferred on top of 2 ml Fico/Lite LymphoH in a 13 ml tube (100 x 16 mm) and centrifuged at 2000 x g for 30 min. Mononuclear cells (85-95% lymphocytes) were then collected from the Fico/Lite interface, transferred to 2 ml red cell lysis buffer for 20 sec and diluted with 4 ml Dulbecco's phosphate buffered saline (DPBS) at twice the normal strength. Lymphocytes were centrifuged at 1000 x g for 5 min and resuspended with modified TCM-199 [TCM-199 supplemented with 5% (v/v) horse serum, 200 units/ml penicillin, 0.2 mg/ml streptomycin, 1 mmol l^{-1} glutamine and 10^{-5} mol l^{-1} β-mercaptoethanol]. Lymphocyte number was determined using hemocytometer and adjusted to 1 x 10^6 cells/ml. Lymphocytes were then assigned to treatments and cultured in modified TCM-199 to determine heat-induced apoptosis and intracellular GSH.

Lymphocyte Apoptosis

Effects of heat shock on apoptosis was evaluated for lymphocyte preparations from 8 cows from each breed. For each cow, a total of 1-5 ml lymphocytes were cultured in 100 x 16 mm sterile tubes at 38.5°C for 24 h or at 41°C for 9 h followed by 15 h at 38.5°C. After cultures, lymphocytes were centrifuged for 10 min at 1000 x g and the pellet resuspended in 5 ml PBS-PVP. Lymphocytes were centrifuged again and the pellet was resuspended and incubated in 5 ml of 4% (w/v) paraformaldehyde in 100 μmol l^{-1} phosphate with 0.9% (w/v) NaCl for 1 h at room temperature. Lymphocytes were then washed in PBS-PVP by centrifugation and resuspended in 1 ml of PBS-PVP. A 100 μl aliquot of lymphocyte suspension was transferred to a poly-l-lysine coated slide and allowed to dry for at least 24 h at room temperature. Slides were stored at 4°C until TUNEL staining.

For TUNEL staining, slides were washed twice in PBS-PVP (2 min/wash) and cells permeabilized with 0.5% (v/v) Triton X-100 containing 0.1% (w/v) sodium citrate for 1 h at room temperature. Positive controls were incubated with RQ1 RNase-free DNase (50 units/ml) at 37°C for 1 h. Lymphocytes were washed in PBS-PVP and incubated with 50 μl TUNEL reaction mixture (containing fluorescein isothiocyanate-conjugated dUTP and the enzyme terminal deoxynucleotidyl transferase as prepared by the manufacturer) for 1 h at 37°C in the dark. Negative controls were incubated in the absence of terminal deoxynucleotidyl transferase. Lymphocytes were then incubated with RNase A (50 µg/ml) for 1 h at room temperature, followed by 0.5 g/ml propidium iodide for 30 min at room temperature. Lymphocytes were washed 4 times in PBS-PVP to remove excess propidium iodide and coverslips mounted with mounting medium containing Prolong Antifade® as recommended by the manufacturer. Slides were observed using a Zeiss Axioplan 2 fluorescence microscope with dual filter. Percent apoptotic lymphocytes were determined by counting total number of nuclei and total number of TUNEL-labeled nuclei in 100 cells at total of 10 different sites on the slide.

Lymphocyte Glutathione Content

Effects of heat shock on glutathione content was evaluated for lymphocyte preparations from 16 (Angus, Holstein, and Brahman) or 7 (Senepol) cows from each breed. A total of 1-5 ml lymphocytes were cultured in 100 x 16 mm sterile tubes at 38.5°C or 41°C for 9 h. At the end of culture, lymphocytes were centrifuged at 1000 x g for 10 min. The supernatant was discarded and cells were diluted with 1 ml deionized water. After 2 min, samples were centrifuged for 5 min at 1000 x g and the supernatant was stored at - 20°C until assay for glutathione.

Glutathione content was assayed in duplicate using a previously described assay (Baker et al., 1990) based on the reduction of DTND [5,5-dithiobis(2-nitrobenzoic acid)] to TND [5-thio-2-nitrobenzoic acid] using GSH. Fifty microliters of sample or standards (1.6875-200 pmol/well) were pipetted into duplicate wells of a 96-well microtiter plate. This was followed by addition of 100 µl of a freshly prepared reaction mixture containing 5 ml 1 mmol l^{-1} DNTB, 5 ml 1 mmol l^{-1} NADPH, 5.75 ml 100 mmol l^{-1} $NaPO_4$ buffer, pH 7.5 with 1 mmol l^{-1} EDTA and 0.1 ml 200 U/ml glutathione reductase. Absorbance at 405 nm of each well of the 96-well plate was determined at 2 min intervals using a microtiter plate reader (BIO-TEK Instruments, INC., model EL 309).

Statistical Analysis

Data were analyzed by least-squares analysis of variance using the Mixed Models procedure of SAS (SAS, 1989). Percentage data were transformed using the arcsin transformation before analysis. Independent variables varied according to experimental design. The mathematical model included main effects and all interactions. For variables in which each cow was measured more than once, effect of cow nested within breed was considered random and all the other main effects were fixed. Tests of significance were conducted using appropriate error terms as determined by calculating expected mean squares. Effects of breed and breed x temperature were partitioned into individual orthogonal contrasts with the following breed comparisons: Brahman and Senepol vs Angus and Holstein; Brahman vs Senepol; and Angus vs Holstein. Percentage of embryos developing to the blastocyst stage was analyzed with and without Senepol in the data set.

Results

Breed Differences in Resistance of Embryos to Heat Shock

There was no difference in the proportion of cows that ovulated following estrous synchronization (Figure 5-1A) or number of oocytes recovered per ovary among breeds (Figure 5-1B). However, there was a breed effect for cleavage rate ($p < 0.05$). Cleavage rate tended ($p = 0.07$) to be higher for Brahman than other breeds (Figure 5-1C).

On day 4 after insemination, stage of embryonic development for cleaved embryos was visually assessed for each replicate and determined in a total of 1305 embryos (170-587 embryos/breed). There was no difference among breeds on stage of embryonic development at day 4 after insemination (Brahman= 8.38 + 0.50 cells; Holstein= 8.05 + 0.91 cells; Angus= 7.68 + 1.2 cells and Senepol= 8.90 + 1.01 cells). Therefore, embryos from different breeds were at the same developmental stage when they were selected for treatments. Embryos derived from Senepol oocytes had very low development to the blastocyst stage at 38.5°C and it was not possible to determine heat shock effects. Therefore, data from Senepol embryos were removed from the final analysis. Exposure of embryos > 8 cell stage to heat shock of 41°C for 6 h reduced development to the blastocyst stage on day 8 ($p< 0.001$; Figure 5-2A) and 9 ($p< 0.001$; Figure 5-2B) after insemination. However, the negative effect of heat shock on development of embryos to the blastocyst stage was less pronounced for Brahman embryos than for Holstein or Angus embryos on day 8 (breed x temperature, $p< 0.05$; Figure 5-2A) and day 9 (breed x temperature, $p< 0.05$; Figure 5-2B). The proportion of embryos that became expanded or hatched blastocysts on day 9 after insemination was also reduced ($p< 0.001$) by heat shock and the deleterious effects of heat shock was less pronounced in Brahman embryos than Holstein and Angus embryos (breed x temperature, $p< 0.05$; Figure 5-2C). Moreover, heat shock reduced total number of cells per embryo for Holstein and Angus embryos but not for Brahman (breed x temperature, $p< 0.05$; Figure 5-2D).

Oviductal and Endometrial Explants

Culturing oviductal explants at 41°C for 9 h (Figure 5-3) or endometrial explants at 41°C for 3 or 9 h (Figure 5-4) had no effect on incorporation of [^{3}H]leucine into newly synthesized proteins secreted into the medium. In general there were no breed effects except that oviductal protein secretion tended to be higher for Brahman than the other breeds (Brahman vs Senepol; $p < 0.07$).

Lymphocyte Apoptosis and GSH Content

As shown in Figure 5-5, exposure of bovine lymphocytes to 41°C for 9 h increased ($p < 0.001$) the proportion of apoptotic cells. There was, however, a trend for a breed x temperature interaction ($p = 0.07$; Figure 5-5). Further analysis revealed that the (Brahman + Senepol vs Angus + Holstein) x temperature interaction was significant ($p < 0.05$) but that there were no interactions with temperature for Brahman vs Senepol and Angus vs Holstein. Thus, heat-induced apoptosis is less for Brahman and Senepol than for Angus and Holstein. Differences between Brahman and Senepol in the effect of heat shock were difficult to interpret because the degree of apoptosis at 38.5°C was higher for Senepol than Brahman. Accordingly, the effect of heat shock also was examined by calculating for each cow the absolute heat-induced increase in apoptosis (i.e., percent apoptotic at 41°C minus percent apoptotic at 38.5°C). By this criterion, the increase in apoptosis caused by heat shock was greater ($P < 0.05$) for Angus (6.4 + 1.3%) and Holstein (4.1 + 1.4%) than for Brahman (1.9 + 1.3%) and Senepol (1.9 + 1.4%) but were similar for Brahman and Senepol and for Angus and Holstein. In another experiment, heat shock of 41°C for 9 h reduced ($p < 0.01$) intracellular GSH content in lymphocytes but there were no effects of breed or breed x temperature (Figure 5-6).

Discussion

Exposure to heat stress has less deleterious effects on fertility of breeds of cattle adapted to hot climates than breeds from temperate climates (Turner, 1982; Madalena et

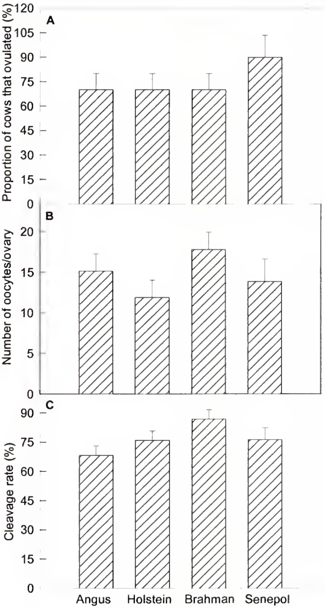

Figure 5-1. Breed differences on the proportion of cows that ovulated after estrous synchronization, oocyte recovery and cleavage rate following in vitro maturation and fertilization. Results are least-squares means ± SEM. There was no difference among breeds on the proportion of cows that ovulated (Panel A) or number of oocytes recovered per ovary (Panel B). However, there was a breed effect for cleavage rate (Panel C; $p< 0.05$). Cleavage rate was higher for Brahman than for the other breeds (Brahman and Senepol vs others, $p= 0.07$; Brahman vs Senepol, $p= 0.07$)

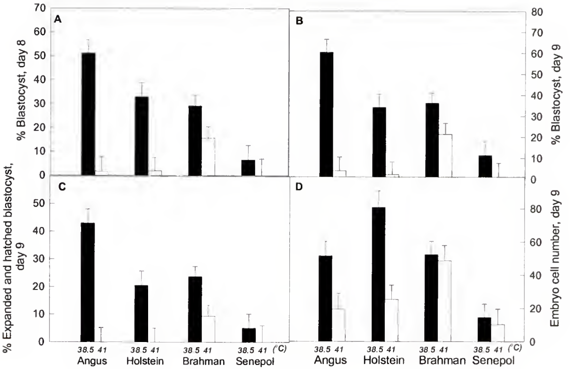

Figure 5-2. Effect of breed on the action of heat-shock on embryonic development. Results are least-squares means ± SEM. Because of the low development of Senepol, data were analyzed excluding this breed. Heat shock of 41°C for 6 h reduced the percentage of embryos that developed to the blastocyst stage on day 8 ($p < 0.001$, panel A) and day 9 ($p < 0.001$, panel B) after insemination, the proportion of embryos that became expanded or hatched blastocysts ($p < 0.001$, panel C) and total embryo cell number ($p < 0.01$, panel D). There was a breed x temperature interaction for each of these variables ($p < 0.05$ for percent blastocyst at day 8 and day 9 and $p < 0.05$ for percent embryos that were expanded or hatched blastocysts at day 9 and total cell number). These interactions reflected the fact that the negative effect of heat shock was less pronounced for Brahman embryos than for Angus and Holstein embryos (Brahman vs others x temperature; $p < 0.05$ for each variable) and because responses differed between Angus and Holstein (Angus vs Holstein) x temperature; $p=0.05$ for percent blastocyst, percent expanded or hatched blastocysts at day 9 and total cell number)

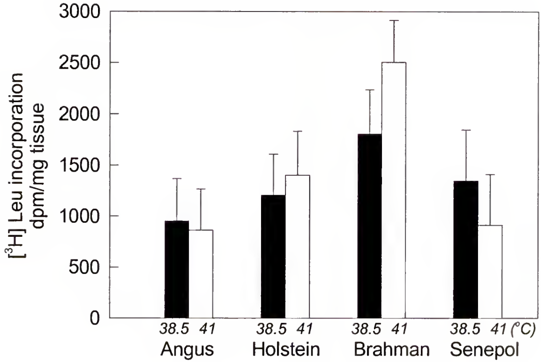

Figure 5-3. Breed differences in heat-shock induced changes in incorporation of [^{3}H]leucine into TCA-precipitable protein secreted into the medium of oviductal explants. Results are least-squares means ± SEM. There was no effect of temperature. There were also no breed effects except that oviductal protein secretion tended to be higher for Brahman than the other breeds (Brahman vs Senepol; $p < 0.07$).

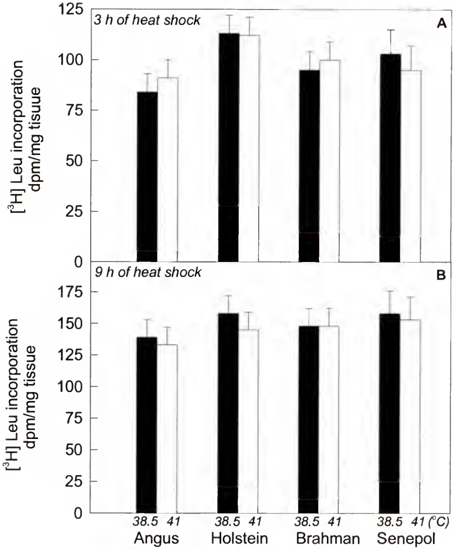

Figure 5-4. Breed differences in heat-shock induced changes in incorporation of [^{3}H]leucine into TCA-precipitable protein into the medium of endometrial explants after culture for 3 (Panel A) or 9 h (Panel B). Results are least-squares means ± SEM. There was no effect of breed or temperature.

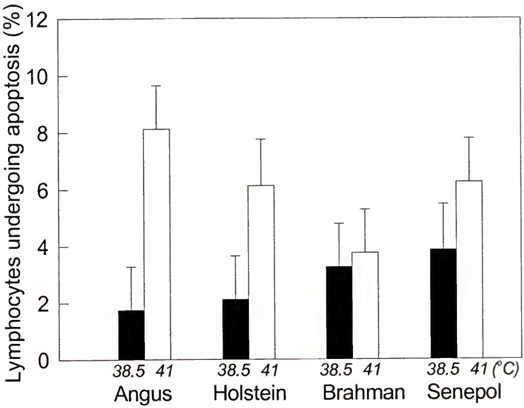

Figure 5-5. Effect of breed on heat-induced apoptosis in lymphocytes. Results are least-squares means ± SEM. Exposure of lymphocytes to 41°C for 9 h increased ($p < 0.001$) the proportion of cells undergoing apoptosis as determined by the TUNEL procedure. There was a breed x temperature interaction ($p = 0.07$). Separation of this interaction into individual degree-of-freedom, orthogonal contrasts revealed that Brahman and Senepol responded to heat shock differently than Angus and Holstein [(Brahman + Senepol vs Angus + Holstein) x temperature, $p < 0.05$] but that responses were similar for Brahman vs Senepol and Angus vs Holstein (i.e., interactions were not significant).

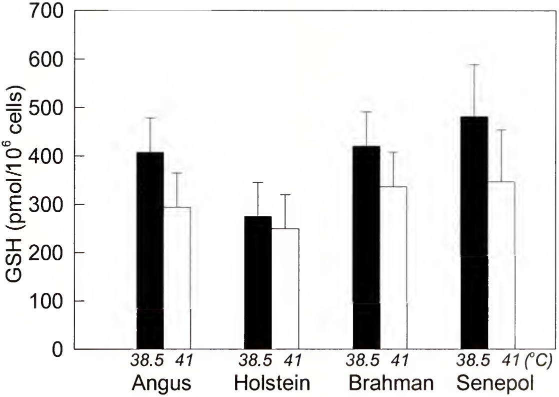

Figure 5-6. Breed differences in heat-shock induced changes in intracellular GSH content of lymphocytes. Results are least-squares means ± SEM. Heat shock reduced ($p < 0.01$) intracellular GSH in lymphocytes but there was no effect of breed or breed x temperature.

al., 1990; Rocha et al., 1998). This breed difference has been attributed to the superior ability of heat-tolerant cattle to regulate body temperature in the presence of heat stress. Here it was demonstrated that genetic adaptations exists at the cellular level as well, and that Brahman and Senepol cattle have evolved to acquire mechanisms to protect cells against the deleterious effects of high temperature. In particular, embryos from Brahman cattle were better able to survive after exposure to elevated temperature than embryos from Angus and Holstein, two heat sensitive breeds of cattle. Additionally, lymphocytes from Brahman and Senepol cattle experienced less apoptosis following heat shock than lymphocytes from the other breeds. Thus, Brahman and Senepol cattle cannot only regulate body temperature in the face of heat stress more effectively than breeds from temperate climates (Hammond et al., 1996; Hammond et al., 1998; Gaughan et al., 1999), but hyperthermia that does result from heat stress is less likely to have severe effects on cellular function. Natural selection for traits that confer cellular resistance to high or low temperature has been described for ectothermic species (Johnston and Walesby, 1977; Crawford and Powers, 1989; Behan-Martin et al., 1993; Somero et al., 1996). The finding that breeds of cattle differ in cellular thermotolerance is, to our knowledge, the only case where closely-related homeotherms have been shown to have undergone such genetic divergence.

Differences in cellular thermotolerance between tissues from Brahman cattle vs Holstein and Angus cattle have been described previously for reproductive tract tissues (Malayer and Hansen, 1990a) and lymphocytes (Kamwanja et al., 1994). The present observation that this genetic difference exists in the preimplantation embryo makes this phenomenon physiologically relevant to embryonic mortality caused by heat stress.

While heat shock of 43°C can compromise oviductal and endometrial function (Malayer et al., 1988; Putney et al., 1988a; Malayer and Hansen, 1990a) , this temperature is much higher than would be experienced by a heat-stressed cow (39.5-41°C). At more physiologically-relevant temperatures, such as the 41°C used here, oviductal and endometrial tissue were not affected by heat shock. In contrast, as shown in this paper and elsewhere (Rivera and Hansen, 2001; Al-Katanani and Hansen, 2002), exposure of embryos to 41°C inhibits embryonic development. Identification of the genes controlling cellular thermotolerance in Brahman cattle may, therefore, lead to genetic strategies for improving embryonic survival during heat stress in breeds that are not thermotolerant. An important question will be to know how early in development genetic differences in embryonic resistance to heat shock exist. Bovine embryos are most sensitive to heat shock in the first few cleavage divisions (Edwards and Hansen, 1997). Since this period is also one of limited gene expression (Memili and First, 2000), it is possible that genetic differences in thermotolerance will not be expressed until after embryonic genome activation is fully developed, i.e., at the 8-16 cell stage in bovine embryos.

Like the Brahman, the Senepol is a breed that is adapted for hot climates (Hammond et al., 1996; Gaughan et al., 1999) although it is *B. taurus* instead of *B. indicus*. In a previous study, Senepol lymphocytes were more resistant to the lethal effects of exposure to 42 or 45°C than lymphocytes from Angus cows (Kamwanja et al., 1994). Present results are consistent with this earlier finding since the effect of heat shock on the percentage of cells undergoing apoptosis was less for Brahman and Senepol than for Angus and Holstein. Moreover, the change in percent apoptotic cells caused by heat shock was similar for Senepol and Brahman. Unfortunately, results were not conclusive regarding whether embryos from Senepol are more resistant to heat shock than embryos from Angus and Holstein because the low development of Senepol embryos at 38.5°C precluded a test of heat shock effects on development in this breed. The reason for low development in the Senepol is not known and could include conditions for freezing spermatozoa, oocyte maturation, or embryo culture that are inappropriate for that breed.

Little is known about the biochemical basis for cellular resistance to elevated temperature in Brahman or Senepol cattle. Heat shock can cause increased free radical metabolism (Loven, 1998) but the lack of breed effect on the reduction in GSH content caused by heat shock does not support the idea that Brahmans have a different antioxidant status than the other breeds tested. Given the complexity of free radical biology, additional studies on this topic are warranted. There is also no evidence for genetic differences in heat shock protein 70 responses between lymphocytes from Brahman, Angus and Senepol cows (Kamwanja et al., 1994). Present results do indicate that Brahman and Senepol lymphocytes were less likely to undergo apoptosis in response to heat shock than lymphocytes from Angus and Holstein. Perhaps the signal that leads to heat-induced apoptosis is dampened in Brahman and Senepol (for example, activation of membrane acid sphingomyelinase (Haimovitz-Friedman et al., 1997; Pena et al., 1997) or the ratio of pro-apoptotic to anti-apoptotic modulatory proteins is different in Brahman and Senepol lymphocytes than in lymphocytes from Angus and Holstein. Before the significance of breed differences in apoptosis can be understood, it needs to be established whether cells from Brahman and Senepol are more resistant to heat shock because they do not die through apoptosis or if they do not undergo apoptosis because cellular physiology is not compromised by heat shock.

One of the features of reproductive function in mammals is the large impact of the environment on reproductive success (Thatcher and Hansen, 1993). Given this fact, and the crucial importance of reproduction in natural selection, it is not surprising that natural selection has caused divergence in the degree to which environmental stimuli can alter reproductive function. Such divergence has been demonstrated in rodents for control of reproduction by photoperiod (Dark et al., 1983). Present results provide evidence that there is also genetic divergence in response of embryonic tissues to heat shock. The importance of this genetic adaption for determining superior reproductive function of Brahman cattle during heat stress (Turner, 1982; Madalena et al., 1990; Rocha et al., 1998) probably depends upon the magnitude of heat stress. Certainly, the superior ability of the Brahman to maintain a constant body temperature under high environmental temperature climates (Hammond et al., 1996; Hammond et al., 1998; Gaughan et al., 1999) and the greater ability of Brahman embryos to function and develop after such heat shocks could contribute to the superior reproductive performance of the Brahman during heat stress.

CHAPTER 6
FAILURE OF MANIPULATION OF ANTIOXIDANT STATUS TO IMPROVE FERTILITY OF LACTATING HOLSTEIN COWS OR ENHANCE SURVIVAL OF CULTURED EMBRYOS EXPOSED TO HEAT SHOCK

Introduction

Reactive oxygen species are products of natural oxygen metabolism and represent approximately 1-2% of metabolized oxygen (Kowaltowski and Vercesi, 1999; Cadenas and Davies, 2000). The balance between the production and disposal of oxidant molecules is essential for tissue homeostasis. Increased rate of free radical production or decreased rate of removal leads to free radical accumulation and cellular damage (Chan, 1993; Berry and Kohen, 1999). Many antioxidant systems are present in the cell to ensure the removal of free radicals. Among these systems, glutathione peroxidase is a selenium-dependent enzyme located in the cytosol that utilizes the reducing potential of glutathione to reduce hydrogen peroxide and organic peroxides to water (Matés and Sánchez-Jiménez, 1999; Arthur, 2000). Vitamin E (α-tocopherol), which is a strong reducing agent that can give electrons to lipids undergoing peroxidation, is a major antioxidant present in plasma membranes (Kamal-Eldin and Appelqvist, 1996; Wang and Quinn, 2000).

Reproductive consequences of free radical damage include disruption of steroidogenesis (Margolin et al., 1990), spermatozoa (Griveau et al., 1995) and preimplantation embryos (Fujitani et al., 1997). Antioxidant status may be one determinant of reproductive function in dairy cattle. Administration of vitamin E and/or selenium has been reported to reduce the incidence of postpartum reproductive disorders such as retained fetal membranes, metritis, and cystic ovaries (Trinder et al., 1969; Harrison et al., 1984; Aréchiga et al., 1994b) and to improve fertility (Segerson et al., 1977; Shubin, 1986; Aréchiga et al., 1994b; Aréchiga et al., 1998b). In other studies, however, there was no beneficial effect of vitamin E and/or selenium supplementation on reproductive function (Schingoethe et al., 1982; Kappel et al., 1984; Hidiroglou et al., 1987; Ealy et al., 1994; Jukola et al., 1996; Wichtel et al., 1996).

Effects of antioxidants on reproductive function may be more pronounced during heat stress because of the increased metabolic rates associated with cellular hyperthermia. Elevated temperature increases liver peroxidation (Ando et al., 1997) and activity of enzymes involved in free radical production such as cyclooxygenase (Malayer et al., 1990) and xanthine oxidase (Skibba et al., 1989b). Exposure of dairy cows to heat stress decreased total antioxidant activity in blood (Harmon et al., 1997). Like most cells, preimplantation embryos can produce free radicals (Yang et al., 1998). The deleterious effects of heat shock on viability of preimplantation mouse embryos can be reduced by antioxidants including vitamin E (Aréchiga et al., 1994a), GSH (Aréchiga et al., 1994a; Aréchiga et al., 1995) and taurine (Malayer et al., 1992).

The objectives of this series of studies were 1) to determine whether sequential injections of vitamin E and selenium at approximately 21 days before expected calving, 30 and 80 day postpartum improves reproductive function and milk yield of lactating Holstein cows under cool and hot conditions and 2) to test whether vitamin E improves development of in vitro produced bovine embryos exposed to heat shock.

Materials and Methods

Materials

Materials for in vitro fertilization were purchased as described in Chapter 3. Oocyte collection medium was TCM-199 with Hank's salts, without phenol red, and supplemented with 2% (v/v) bovine steer serum (containing 2 U/ml heparin), 100 U/ml penicillin-G, 0.1 mg/ml streptomycin, and 1 mM glutamine. Oocyte maturation medium was TCM-199 with Earle's salts supplemented with 10% (v/v) steer serum, 22 μg/ml sodium pyruvate, 20 μg/ml FSH, 2 μg/ml estradiol 17-β , 50 μg/ml gentamicin and an additional 1 mM glutamine. Modified Tyrode's solutions were obtained from Cell and Molecular Technologies (Lavallette, NJ) to prepare HEPES-TALP, IVF-TALP and Sp-TALP (Parrish et al., 1986). Potassium Simplex Optimized Medium was obtained from Cell and Molecular Technologies. The KSOM, which contains 1 mg/ml BSA, was modified on the day of use by adding an additional 2 mg/ml EFAF-BSA, 2.5 μg/ml gentamicin, essential amino acids (Basal Medium Eagle) and non-essential amino acids (Minimum Essential Medium) purchased from Sigma Chemical Company (St. Louis, MO).

A combination of vitamin E and selenium (MU-SE; Schering-Plough Corp., Kenilworth, NJ) was used for in vivo experiments and vitamin E [(+)-α-tocopherol; Sigma] was used for in vitro studies. Other reagents were purchased from Fisher (Pittsburgh, PA) or Sigma Chemical Company (St. Louis, MO).

Experiments 1-3: Effect of Multiple Vitamin E and Selenium Injections on Fertility of Holstein Cows

For each experiment, cows were randomly assigned to one of two groups. Cows with odd numbered tags received three intramuscular injections of 10 ml MU-SE. The first injection was given at ~ 21 day before expected calving (Experiments 1-2; range 18-24 day) or 8-21 day before expected calving (Experiment 3). For all three experiments, the other two injections were given at ~30 day postpartum (range 27-33 day) and ~80 day postpartum (range 77-83 day). The amount of MU-SE administered was equivalent to 500 mg vitamin E as D- α tocopherol acetate (680 IU) and 109.5 mg of sodium selenite (equivalent to 50 mg selenium). Cows with even numbered tags received injections of placebo [0.9 % (w/v) NaCl] at the same times. For all three experiments, birth difficulty was classified at the time of calving based upon the severity of clinical signs and the need of delivery assistance from 0-5 (0= not observed, 1= no calving difficulty, 2= minor problem, 3= needed assistance, 4= considerable force and 5= very difficult).

Experiment 1 was conducted on a commercial dairy farm in Anthony, Florida (29° 17'N 82 07'W) using 249 Holstein cows. Estrus detection was performed based on visual observation of mounting behavior performed throughout the day and cow locomotor activity using pedometers (AFIMILK System- USA Inc., Visalia, CA). All cows were artificially inseminated after a voluntary waiting period of 90-120 day postpartum, milked three times a day, and placed on treatment with bovine somatotropin (BST, Posilac®, Monsanto, Chesterfield, MO) beginning 60-70 day postpartum as directed by the manufacturer. Pregnancy was determined by rectal palpation at 37 to 52 day after insemination. Beginning 21 day before expected calving, cows were fed a total mixed ration based on chopped hay formulated to provide 12.0 $\text{Mcal}\bullet\text{d}^{-1}\bullet\text{cow}^{-1}$ NE_L , 817 $\text{IU}\bullet\text{d}^{-1}\bullet\text{cow}^{-1}$ vitamin E and 8.5 $\text{mg}\bullet\text{d}^{-1}\bullet\text{cow}^{-1}$ selenium. After calving, cows were fed a total mixed ration based on corn silage and hominy formulated to provide 36.0 Mcal•d^{-1}•cow^{-1} NE$_L$, 412 IU•d^{-1}•cow^{-1} vitamin E and 6.3 mg•d^{-1}•cow^{-1} selenium.

Experiment 2 was conducted at the University of Florida Dairy Research Unit (DRU) (Hague, Florida; 29°46'N 82 25W) using 137 Holstein cows. Estrus detection was based on visual observation of mounting behavior performed throughout the day and evaluation of chalk removal from tailheads. All cows were artificially inseminated after the voluntary waiting period of 70 day postpartum, milked three times a day and BST was utilized as directed by the manufacturer beginning 57-63 day postpartum. Pregnancy was determined by rectal palpation at 42 to 70 day after insemination. The prepartum and postpartum cows were fed a total mixed ration based on corn silage, citrus pulp, corn meal and alfafa hay formulated to provide 35.2 Mcal•d^{-1}•cow^{-1} NE$_L$. The prepartum ration was formulated to provide 530 IU•d^{-1}•cow^{-1} vitamin E and 7 mg•d^{-1}•cow^{-1} selenium and the postpartum ration was formulated to provide 350 IU•d^{-1}•cow^{-1} vitamin E and 7 mg•d^{-1} •cow^{-1} selenium.

Experiment 3 was conducted on a commercial dairy farm in Bell, Florida (29°45'N 82 52'W) using 307 Holstein cows. Estrus detection was performed as described for experiment 1. All cows were artificially inseminated after the voluntary waiting period of 90 day postpartum, milked three times a day and were BST first injected as directed by the manufacturer at 60 day postpartum. Pregnancy was determined by rectal palpation at 42 to 47 day after service. The prepartum cows were fed a total mixed ration based on corn silage, wet brewers' grains and alfafa hay formulated to provide 24.9 Mcal•d-1 •cow^{-1} NE$_L$, 1209 IU•d^{-1}•cow^{-1} vitamin E and 7.5 mg•d^{-1}•cow^{-1} selenium. After calving, cows were fed a total mixed ration based on corn silage and hominy formulated to provide 38.0 Mcal• d^{-1}•cow^{-1} NE_L, 1260 IU•d^{-1}•cow^{-1} vitamin E and 14 mg•d^{-1}•cow^{-1} selenium.

Table 6-1 shows the frequency distribution of month of calving and month of first insemination for all the three experiments. For experiments 1 and 2, most first inseminations after calving were performed during the cool months of the year. For experiment 3, the majority of cows received first insemination in warm months.

Blood Collection and Analysis for Experiments 1-3

Blood samples were collected at the time of first MU-SE injection, 1-2 weeks after calving and every 2 weeks thereafter by venipuncture of the coccygenal vessels. Samples were collected in heparinized tubes, centrifuged for 30 min at 2000 x g at 4°C, and plasma samples were frozen at - 20°C until analysis. Plasma preparation for α-tocopherol analysis using HPLC and the HPLC procedure were as described previously (Njeru et al., 1992). For experiment 1, α-tocopherol was determined in samples from 10 cows treated with vitamin E/selenium (41 samples) and 10 cows treated with placebo (44 samples). For experiment 2, α-tocopherol was determined for samples from 12 vitamin E/selenium treated cows (51 samples) and 5 cows treated with placebo (28 samples). For experiment 3, α-tocopherol was determined for samples from 5 cows treated with vitamin E/selenium (17 samples) and 3 cows treated with placebo (9 samples).

Experiments 4-6: Effects of Vitamin E on Susceptibility of Bovine Embryos to Heat Shock

Procedures for in vitro production of embryos were as described earlier in Chapter 3. Briefly, COCs were obtained by slicing 2-10 mm follicles on the surface of the ovary into a beaker containing oocyte collection medium. Cumulus-oocyte complexes which had at least one layer of compact cumulus cells were washed two times and matured in groups of 10 in 50 μl drops of oocyte maturation medium overlaid with mineral oil for 22 h at 38.5°C in an atmosphere of 5% (v/v) CO_2 in humidified air. For fertilization, groups of ~ 30 COCs were transferred to four well plates containing 600 μl IVF-TALP per well. Oocytes were fertilized with 25 μl sperm suspension and 25 μl PHE [0.5 mM penicillamine, 0.25 mM hypotaurine and 25 M epinephrine in 0.9% (w/v) NaCl] added to each well. Presumptive zygotes were removed from fertilization wells, denuded of cumulus cells, washed 2-3 times in HEPES-TALP to remove remaining cumulus cells and associated spermatozoa, and placed in groups of ~ 25-30 in 50 μl drops of modified KSOM overlaid with mineral oil.

Because vitamin E is hydrophobic, it was first dissolved in absolute ethanol as a 0.2 M stock solution, stored in the dark at 4°C, diluted in modified KSOM to a final concentration of 100 μM vitamin E and 0.05% (v/v) ethanol at 3 h before culture. Embryos were cultured in 50 μl drops of modified KSOM containing either vehicle [0.05% (v/v) ethanol] or 100 μM vitamin E in 0.05% ethanol and overlaid with mineral oil containing the same treatments at 38.5°C and 5% CO_2 (v/v) in humidified air.

Experiments 4 and 5 were each designed with a 2 x 2 factorial arrangement of treatments to determine whether vitamin E protects 2-cell bovine embryos from heat shock. Embryos at the 2-cell stage were harvested 28-30 h after insemination and placed in 50 μl drops. The number of embryos per drop was similar within a replicate. For experiment 4, there were 13-23 embryos per drop while there were 11-21 embryos per drop for experiment 5. Embryos were preincubated for 5 h at 38.5°C in modified KSOM containing either vehicle [0.05% (v/v) ethanol] or 100 μM vitamin E in 0.05% (v/v) ethanol. Following preincubation, embryos were either maintained at 38.5°C continuously or exposed to a heat shock of 41°C for 9 h (experiment 4) or 4.5 h (experiment 5). The percentage of embryos developing to the blastocyst stage in each drop was determined on day 8 after insemination. Experiment 4 was replicated 4 times using 114-121 embryos/group (1-2 drops of embryos per treatment in each replicate). Experiment 5 was replicated 4 times using 92-137 embryos/group (1-2 drops of embryos per treatment in each replicate).

Experiment 6 utilized a 2 x 2 factorial arrangement of treatments to determine whether vitamin E protects bovine embryos at the morula stage from heat shock. Embryos at the morula stage were harvested on day 5 after insemination, placed in 50 μl drops (10-23 embryos per drop; the number of embryos per drop were similar for all treatments within a replicate) and treated as for experiment 4. The percentage of embryos developing to the blastocyst stage in each drop was determined on day 8 after insemination. The experiment was replicated 4 times using 125-183 embryos/group (1-3 drops of embryos per treatment was prepared for each replicate).

Statistical Analyses

Data were analyzed by least-squares analysis of variance using the GLM procedure and the Mixed Models procedure of SAS (SAS, 1989). Initial analysis of the reproductive data for experiment 1 and 2 included the main effects of treatment, parity (primiparous vs multiparous) and season of first breeding (cool season- cows bred from September to April; hot season- cows bred from May to August) as well as interactions between these terms. Initial analysis of data for experiment 3 included main effects of treatment, parity (primiparous vs multiparous), season of first breeding (cool season- cows bred from September to April; hot season- cows bred from May to August) and the time of the prepartum injection relative to calving (cows injected > 14 days before expected calving; cows injected < 14 days and > 8 days before expected calving; cows injected < 8 days before expected calving). Non-significant terms were subsequently removed from the analysis. Thus, the mathematical model for the final analysis of reproductive data for experiments 1-3 included main effects of treatment and parity. The mathematical model for data on milk yield and composition included main effects of treatment, parity and month of calving, all interactions, and with days in milk as a covariate. Plasma α-tocopherol concentration data were analyzed using main effects of treatment, time of sample collection relative to calving (prepartum, and 1-14, 15-28, 29-35, 36-42 and > 42 day postpartum), and cow. Effect of cow was considered a random effect and all other main effects were fixed.

For embryo studies, there was one or more drop per treatment for each replicate and the percentage of embryos developing to the blastocyst stage was calculated for each drop. Percentage data were transformed using the arcsin transformation before analysis. Data were analyzed using main effects of treatment, temperature and replicate using the GLM procedure of SAS, where replicate (i.e., day of IVF procedure) was considered a fixed variable, and by the Mixed Models procedure of SAS, where replicate was considered a random variable (SAS, 1989). Probability values were similar for both methods and probability values reported herein are derived from the analysis by GLM.

Results

Effect of Multiple Vitamin E and Selenium Injections on Fertility and Milk Yield

Intramuscular injection of 500 mg vitamin E and 50 mg selenium before calving and at 30 and 80 day postpartum did not affect plasma α-tocopherol concentrations in

Table 6-1. Frequency distribution of cows calving and first inseminations by month.[a]

Month of calving	Experiment 1		Experiment 2		Experiment 3	
	calving	inseminations	calving	inseminations	calving	inseminations
January	0 (0.0%)	61 (24.5%)	0 (0.0%)	24 (17.7%)	1 (.3%)	0 (0.0%)
February	0 (0.0%)	65 (26.1%)	1 (0.7%)	16 (11.8%)	0 (0.0%)	0 (0.0%)
March	0 (0.0%)	14 (5.6%)	1 (0.7%)	18 (13.2%)	41 (13.4%)	1 (0.4%)
April	0 (0.0%)	1 (0.4%)	0 (0.0%)	1 (0.7%)	65 (21.2%)	0 (0.0%)
May	2 (0.80%)	0 (0.0%)	0 (0.0%)	0 (0%)	101 (32.9%)	4 (1.4%)
June	21 (8.4%)	0 (0.0%)	2 (1.4%)	1 (0.7%)	92 (30.0%)	17 (6.1%)
July	32 (12.9%)	0 (0.0%)	10 (7.3%)	0 (0.0%)	4 (1.3%)	90 (32.0%)
August	22 (8.8%)	0 (0.0%)	29 (21.0%)	1 (0.7%)	2 (0.7%)	77 (27.4%)
September	66 (26.5%)	25 (10.0%)	25 (18.8%)	8 (5.9%)	1 (0.3%)	47 (16.7%)
October	54 (21.7%)	13 (5.2%)	11 (8.0%)	30 (22.1%)	0 (0.0%)	41 (14.6%)
November	52 (20.9%)	21 (8.4%)	37 (25.8%)	18 (13.2%)	0 (0.0%)	2 (0.7%)
December	0 (0.0%)	49 (19.7%)	21 (15.2%)	19 (14.0%)	0 (0.0%)	2 (0.7%)

[a] Data represent numbers of cows and, in parentheses, the percentage of total cows in the experiment.

any of the three experiments (Table 6-2). In experiment 1, in which most first inseminations were in the cool season, administration of vitamin E and selenium did not improve the proportion of cows that became pregnant by 180 day postpartum. Also, there was no beneficial effect of vitamin E and selenium on days from calving to first service, number of services by 180 day postpartum, birth difficulty or predicted 305-d milk yield. Similar results were also obtained for experiment 2, where most first inseminations were also in the cool season. As shown in Table 6-4, vitamin E and selenium injections did not improve pregnancy rate at first service or the proportion of cows that became pregnant by 120 day postpartum. Vitamin E and selenium injections did not affect days from calving to first service, number of services by 120 day postpartum, birth difficulty or any of the milk yield traits examined.

Experiment 3 was conducted with most first inseminations in the hot season. In this experiment, vitamin E and selenium injection did not affect pregnancy rate at first or second service or at 90, 120 or 150 day postpartum (Table 6-5). Also, there was no beneficial effect of vitamin E and selenium on days from calving to first service, number of services by 120 day postpartum, birth difficulty, body condition score or any of the milk yield traits examined.

Effects of Vitamin E on Susceptibility of Bovine Embryos to Heat Shock

In experiment 4, exposure of 2-cell bovine embryos to heat shock of 41°C for 9 h reduced development to the blastocyst stage (Figure 6-1B; $p< 0.001$). However, culture of embryos in medium containing 100 μM vitamin E did not alter development at 38.5°C or 41°C or reduce the deleterious effects of 41°C on development. When embryos at the 2-cell stage were exposed to a milder heat shock of 41°C for 4.5 h (experiment 5), there was also a deleterious effects of heat shock (Figure 6-1A; $p < 0.05$) and no beneficial effect of vitamin E on development. In this study, culture of embryos in medium containing 100 μM vitamin E tended to reduce development at 38.5°C (treatment x temperature, $p = 0.09$). In contrast to the deleterious effects of heat shock on 2-cell embryos, exposure of morulae to heat shock of 41°C for 9 h did not decrease development to the blastocyst stage (Figure 6-1C). Culture in medium containing 100 μM vitamin E had no beneficial effect on blastocyst formation at either temperature. For all the three experiments, there was no effect of vitamin E on the percentage of blastocysts that became expanded or hatched blastocyst (data not shown).

Discussion

Multiple vitamin E and selenium injections failed to improve reproductive function or milk yield in lactating Holstein cows. In this respect, results are in agreement with others in which injection of vitamin E, selenium or vitamin E and selenium failed to affect reproductive function (Schingoethe et al., 1982; Kappel et al., 1984; Hidiroglou et al., 1987; Ealy et al., 1994; Jukola et al., 1996; Wichtel et al., 1996) or milk yield (Campbell and Miller, 1998; Shin et al., 1999; Baldi et al., 2000). In contrast, other studies have reported a beneficial effect of supplemental vitamin E or vitamin E and selenium on fertility (Segerson et al., 1977; Shubin, 1986; Aréchiga et al., 1994b; Aréchiga et al., 1998b) and milk yield (Lacetera et al., 1996). Much of the variation in results among studies may be due to differences in dietary content of vitamin E and selenium as well as timing, dosage and method of administration of supplemental vitamin E and selenium. For example, the amounts of vitamin E and selenium fed to the prepartum cows in the present study (530-1209 $IU \cdot d^{-1} \cdot cow^{-1}$ of vitamin E and 7-14

Table 6-2. Concentrations of plasma α-tocopherol as affected by multiple vitamin E and selenium injections [a]

Time postpartum (days)	Plasma α-tocopherol concentration (μg/ml)					
	Experiment 1		Experiment 2		Experiment 3	
	Control	Vitamin E/ selenium	Control	Vitamin E/ selenium	Control	Vitamin E/ selenium
Prepartum[b]	2.83 ± 0.87	3.20 ± 0.97	5.00 ± 2.04	5.00 ± 1.70	3.41 ± 0.64	4.36 ± 0.50
1-14	3.10 ± 1.10	1.66 ± 1.56	0 ± 4.25	3.20 ± 2.50	.	.
15-28	3.82 ± 2.02	1.80 ± 1.43	9.90 ± 3.50	5.04 ± 2.50	.	.
29-35	3.00 ± 1.43	2.85 ± 2.47	11.71 ± 3.03	6.00 ± 2.70	4.36 ± 0.64	4.31 ± 1.08
36-42	3.72 ± 1.75	4.06 ± 1.75	9.00 ± 4.25	7.65 ± 2.30	.	.
> 42	8.85 ± 1.05	9.86 ± 0.87	19.30 ± 2.04	20.06 ± 1.70	6.00 ± 0.66	6.00 ± 0.41

[a] Data represent least square means ± SEM.

[b] Prepartum samples were taken on the day of first vitamin E and selenium injection

Table 6-3. Effect of multiple vitamin E and selenium administration on reproductive function and milk yield of lactating Holstein cows (Experiment 1) [a]

Reproductive and milk yield traits	Control	Vitamin E/selenium
Birth difficulty	1.6 ± 0.08	1.6 ± 0.08
Interval from calving to first service, days	106.0 ± 2.0	103.2 ± 2.0
Pregnant at first service, % [b]	31.0 ± 5.7 (40/125)	33.2 ± 5.6 (35/122)
Pregnant at second service, % [b]	31.0 ± 5.0 (21/68)	26.6 ± 5.1 (23/69)
Pregnant at 180 days postpartum, % [b]	61.1 ± 4.5 (74/120)	60.0 ± 4.5 (71/119)
Number of services at 180 days postpartum	2.0 ± 0.10	2.0 ± 0.10
Days in milk, last test day	293.0 ± 6.1	272.5 ± 6.1
Last test day milk yield, lbs	21984 ± 266.0	21833 ± 261.0
Predicted 305-d milk yield, lbs	21242 ± 344.2	20551 ± 337.8

[a] Cows were either injected with 500 mg vitamin E and 109.5 mg sodium selenite or placebo at 21 days before expected calving, 30 and 80 days postpartum. Data represent least square means ± SEM.

[b] Numbers in parentheses represent number of pregnant cows per number of total cows.

Table 6-4. Effect of multiple vitamin E and selenium administration on reproductive function and milk yield of lactating Holstein cows (Experiment 2) [a]

Reproductive and milk yield traits	Control	Vitamin E/selenium
Birth difficulty	1.5 ± 9.0	1.6 ± 8.5
Interval from calving to first service, days	72.6 ± 2.3	70.2 ± 2.2
Pregnant at first service, % [b]	17.0 ± 5.0 (9/66)	21.1 ± 4.6 (13/72)
Pregnant at 120 days postpartum, % [b]	35.8 ± 6.3 (21/65)	39.4 ± 6.0 (25/71)
Number of services at 120 days postpartum	1.8 ± 0.12	1.7 ± 0.11
Days in milk, last test day	295.1 ± 7.6	290.6 ± 8.0
Last test day milk yield, lbs	16985 ± 840.5	16586 ± 846.0
Last test day fat, lbs	572.5 ± 32.2	575.4 ± 32.4
Last test day protein, lbs	535.8 ± 24.0	529.2 ± 24.2
Predicted 305-d milk yield, lbs	15872 ± 1382.1	15312 ± 1424.0

[a] Cows were either injected with 500 mg vitamin E and 109.5 mg sodium selenite or placebo at 21 days before expected calving, 30 and 80 days postpartum. Data represent least square means ± SEM.

[b] Numbers in parentheses represent number of pregnant cows/number of total cows.

Table 6-5. Effect of multiple vitamin E and selenium administration on reproductive function and milk yield of lactating Holstein cows (Experiment 3) [a]

Reproductive and milk yield traits	Control	Vitamin E/selenium
Birth difficulty	1.3 ± 5.6	1.4 ± 6.0
Interval from calving to first service, days	92.4 ± 2.0	88.8 ± 2.0
Pregnant at first service, % [b]	26.2 ± 4.0 (31/118)	21.0 ± 4.1 (22/105)
Pregnant at second service, % [b]	31.0 ± 5.0 (26/84)	26.6 ± 5.1 (21/79)
Pregnant at 90 days postpartum, % [b]	16.5 ± 3.2 (20/121)	14.0 ± 3.4 (15/107)
Pregnant at 120 days postpartum, % [b]	27.1 ± 4.2 (32/108)	32.0 ± 4.4 (34/106)
Pregnant at 150 days postpartum, % [b]	47.4 ± 4.6 (55/116)	46.1 ± 5.0 (48/104)
Number of services at 120 days postpartum	1.8 ± 0.12	1.7 ± 0.11
Days in milk, last test day	251.0 ± 2.2	251. 2 ± 2.5
Last test day milk yield, lbs	20334 ± 200.0	20546 ± 208.7
Last test day fat, lbs	707.7 ± 8.1	718.2 ± 8.5
Last test day protein, lbs	589.0 ± 5.3	600.0 ± 5.5
Predicted 305 milk yield, lbs	22387 ± 213.5	22606 ± 223.3

[a] Cows were either injected with 500 mg vitamin E and 109.5 mg sodium selenite or placebo at 21 days before expected calving, 30 and 80 days postpartum. Data represent least square means ± SEM.

[b] Numbers in parentheses represent number of pregnant cows/number of total cows.

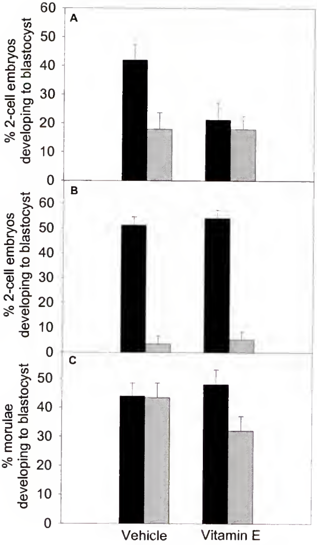

Figure 6-1. Lack of vitamin E effect on development of bovine embryos exposed to heat shock. Results are least-squares means ± SEM. Exposure of 2-cell embryos to heat shock of 41°C for 4.5 (panel A; $p< 0.05$) or 9 h (panel B; $p< 0.001$) reduced development to the blastocyst stage. Addition of 100 µM vitamin E to the culture medium of 2-cell embryos did not reduce the deleterious effect of 41°C on development. However, culture of 2-cell embryos in medium containing 100 µM vitamin E tended to reduce development at 38.5°C (treatment x temperature, $p= 0.09$) in experiment 5 (panel A) but not in experiment 4 (Panel B). Heat shock of 41°C for 9 h did not decrease development of morulae to the blastocyst stage and addition of 100 µM vitamin E had no beneficial effect on blastocyst formation at either temperature (panel C). Black bars are embryos exposed to 38.5°C and grey bars are embryos exposed to 41°C.

$mg \bullet d^{-1} \bullet cow^{-1}$ selenium) were higher than the 500 $IU \bullet d^{-1} \bullet cow^{-1}$ vitamin E and 5 $mg \bullet d^{-1} \bullet cow^{-1}$ selenium for two studies in Mexico in which vitamin E and selenium injections increased fertility (Aréchiga et al., 1994b; Aréchiga et al., 1998b). The dose of vitamin E injected into treated cows was less than the amount of vitamin E supplied in two days's ration and this dose may not be effective at altering chronic vitamin E status. In support of this idea is the observation that there was no effect of vitamin E and selenium injections on plasma concentrations of α-tocopherol measured every two weeks after injection. Others have also failed to observe effects of injection of vitamin E at higher concentrations than used here on plasma concentrations of α-tocopherol (Weiss et al., 1997; Bass II et al., 2000). In contrast, the amount of selenium injected was probably sufficient to affect antioxidant status since intramuscular injection of selenium can increase glutathione peroxidase activity for at least 3 weeks (Harrison et al., 1984).

It was reasoned that beneficial effects of vitamin E and selenium injections would be greater in experiment 3, where most of the cows were inseminated in hot weather, than for experiments 1-2, where most of the cows were inseminated in cool weather. The basis for this hypothesis was the idea that the increase in metabolism when cows are in environments above the upper critical temperature would result in increased oxygen consumption and free radical production. Indeed, heat stress has been reported to deplete plasma antioxidant activity (Harmon et al., 1997). Furthermore, the deleterious effects of heat stress on fertility might involve increased free radical production because survival of mouse embryos exposed to elevated temperature could be enhanced by culture in the presence of antioxidants such as glutathione (Aréchiga et al., 1994a; Aréchiga et al., 1995) and vitamin E (Aréchiga et al., 1994a). There was, however, no beneficial effect of vitamin E and selenium in Experiment 3. Pregnancy rates to first service were higher than is typically seen in the summer in Florida and it is possible that cows in this study were less affected by heat stress than is typically the case for lactating cows in Florida because of effective environmental management practices at this farm. In addition, the high dietary content of vitamin E and selenium in experiment 3 may have negated any beneficial effect of injections of these molecules.

Other antioxidant treatments were also without effect on fertility of heat-stressed dairy cows including injection of 3000 IU vitamin E at the time of artificial insemination (Ealy et al., 1994) and multiple injections of β-carotene (Aréchiga et al., 1998a). Feeding supplemental β-carotene to heat-stressed cows had only slight effects on reproductive function (Aréchiga et al., 1998a). Thus, it is likely that the failure of vitamin E and selenium to improve reproductive function during heat stress reflects the fact that antioxidant status is less important for determining reproductive function in the summer than we had hypothesized. Several studies indicate that oxygen consumption of lactating cows decreases during heat stress (Kibler et al., 1949; Kibler and Brody, 1956; Sorensen and Weniger, 1987) probably because feed consumption and milk yield is reduced, and such an effect would decrease free radical production

The present experiments do not support the idea that vitamin E act as an effective thermoprotectant or general antoxidant for cultured bovine embryos. Addition of vitamin E to culture medium did not enhance development of 2-cell bovine embryos or morulae at 38.5°C or exposed to a heat shock of 41°C. In mice, vitamin E reduced the deleterious effects of heat shock on embryonic viability as determined by dye exclusion but did not block the inhibitory effect of heat shock on development to the blastocyst stage (Aréchiga et al., 1994a). Olson and Seidel (2000) observed that vitamin E supplemetation during culture of bovine embryos increased embryonic development to the blastocyst stage. Variation between studies may be due to differences in the system for culturing embryos, i.e., in an atmosphere of 20% oxygen in the present study versus 5% oxygen in the study by Olson and Seidel (2000). It is possible that the high oxygen concentration used in the present study resulted in exacerbated free radical production that could not be compensated for by the use of vitamin E as an antioxidant.

As has been reported earlier (Ealy et al., 1995; Edwards and Hansen, 1997; Krininger III et al., 2002) embryonic sensitivity to heat shock was reduced as embryos advanced in development. In particular, exposure of 2-cell embryos to 41°C reduced the proportion of embryos that developed to the blastocyst stage while a similar heat shock had no effect on development of morula to the blastocyst stage. Thus, embryos acquire resistance to elevated temperature as they advance in development.

In summary, studies failed to yield evidence of a beneficial effect of supplemental vitamin E and selenium above estimated requirements on reproduction or milk yield of lactating dairy cows under cool or heat stress conditions or of vitamin E as an embryonic thermoprotectant or as a culture medium additive for enhancing development of preimplantation embryos in culture. It is concluded that multiple injections of vitamin E and selenium in the manner tested is unlikely to enhance reproductive or lactational performance of lactating dairy cows receiving dietary requirements of these nutrients.

CHAPTER 7
GENERAL DISCUSSION

As stated in the Foreword, the major focus of this dissertation has been to identify determinants of embryonic resistance to elevated temperature. Identification of such mechanisms or markers of thermoresistance might allow the development of novel strategies to protect the bovine embryo from the deleterious effects of elevated temperature and therefore reduce some of the deleterious effects of heat stress on pregnancy rates during summer in hot climates. From the work outlined in this thesis, two of such determinants were identified - the ability of embryos to undergo apoptosis and embryonic genotype. Additionally, experiments presented in this thesis fail to support the idea that antioxidant status is an important determinant of embryonic survival, at least with respect to cows raised under Florida conditions. These results provide information regarding the cellular basis for developmental acquisition of thermotolerance, the evolution of thermotolerance in cattle, and the possible presence of specific genetic polymorphisms in cattle that confer cellular thermotolerance. While the lack of effect of vitamin E and selenium on fertility of lactating dairy cows means that a practical approach for improving dairy cattle fertility was not realized in this thesis, other conclusions derived from the thesis may form the basis for practical approaches for improving embryonic survival during heat stress.

It has long been known that embryos become more resistant to elevated temperature as they advance in development. This developmental acquisition of thermotolerance has been shown in vivo for sheep (Dutt, 1963), pigs (Tompkins et al., 1967), cattle (Putney et al., 1988) and rabbits (Wolfenson and Blum, 1988) and in vitro for cattle (Ealy et al., 1995; Edwards and Hansen, 1997; Ju et al., 1999; Krininger III et al., 2002). One goal of this dissertation was to identify the cellular basis for the development of embryonic resistance to elevated temperature. Evidence from several experiments indicates that embryonic capacity for apoptosis is one developmentally-acquired cellular system that provides embryos with the ability to continue development after heat shock. First, results from Chapter 3 demonstrated that bovine embryos undergo heat-induced apoptosis in a developmentally-regulated manner. Heat shock did not induce apoptosis in embryos at the 2 or 4-cell stage or day 3 embryos at the 8-16 cell stage whereas it induced apoptosis in day 4 embryos at the 8-16 cell stage. Therefore, the 8-16 cell stage seems to be the transition point when bovine embryos acquire ability for heat-induced apoptosis. The timing of heat-induced apoptosis parallels changes in embryonic resistance to heat shock shown by effects of heat shock on embryo cell number in Chapter 3 as well as earlier experiments (Ealy et al., 1995; Edwards and Hansen, 1997; Ju et al., 1999; Krininger III et al., 2002). The congruence between timing of these two events implies that the capacity for apoptosis may be important for embryos to survive heat shock because it allows selective removal of damaged blastomeres without affecting the entire embryo. Experiments in Chapter 4 support this concept since blocking apoptosis with a caspase inhibitor magnified the deleterious effects of heat shock on development. Further research is necessary to determine whether blastocysts formed from embryos that survived heat shock by undergoing apoptosis are able to establish a successful pregnancy. Nonetheless, since embryos that were capable of apoptosis were better able to undergo continued development after heat shock than embryos in which caspase 3 activity was blocked, it is clear that induction of apoptosis by heat shock represents a mechanism that contributes to embryonic thermotolerance.

A model that illustrates the possible role of heat-induced apoptosis on embryonic development is shown in Figure 7-1. According to this model, the fate of the embryo exposed to heat shock depends on the degree of apoptosis. Exposure to a severe heat shock may cause massive apoptosis, a large reduction in embryo cell number, and embryonic death. In contrast, ability to remove damaged cells by apoptosis is beneficial in cases where the heat shock is less severe and apoptosis is limited to a small proportion of blastomeres. In this case, apoptosis my improve embryonic survival by removing the most severely damaged cells. The inability to undergo apoptosis in response to heat shock makes embryos more sensitive to heat shock, for example, as is seen in embryos during the first few cleavage stages (2-8 cell stage) and morulae that are pharmacologically-treated with the caspase inhibitor z-DEVD-fmk. Cells severely damaged by stress that fail to undergo apoptosis often become necrotic (Fuse et al., 1998; Gorman et al., 1999) and caspase inhibition prevented apoptosis and resulted in cell death by necrosis (Hirsch et al., 1997). Necrotic cell death causes cellular swelling, membrane rupture followed by the release of intracellular content and debris which triggers an exudative inflammation (Wyllie et al., 1980). Perhaps embryos unable to undergo apoptosis experience necrosis instead and the entire embryo becomes compromised.

It should also be pointed out that necrosis could also occur in embryos that are capable of necrosis provided the insult is severe enough. In fact, the intensity and severity of cellular damage has been shown to determine whether a cell will die by apoptosis or necrosis (McConkey et al., 1988; Lennon et al., 1991; Formigli et al., 2000).

There are at least two questions that arise from experiments dealing with apoptosis - the mechanisms by which embryos acquire the ability to undergo apoptosis and the practical significance of the relationship between apoptosis and embryonic survival. The acquisition of heat-induced apoptosis, which first occurs somewhere between the 8- and 16-cell stage, is coincident with when the embryonic genome becomes fully activated (Memili and First, 2000). The onset of spontaneous apoptosis in bovine embryos also occurs at the 9-16 cell stage (Byrne et al., 1999; Matwee et al., 2000). Absence of heat-induced apoptosis in embryos at the 2-cell stage is associated with a lack of activation of group II caspases. However, one should be careful in assuming that the early embryo does not undergo caspase activation and apoptosis in response to heat shock because it is lacking some component of the apoptotic pathway and that new transcription is necessary for this component to be replaced. There is evidence that the cell death machinery is constitutively present in early cleavage embryos. The protein kinase inhibitor staurosporine induced caspase activity in mouse zygotes (Exley et al., 1999) and induced apoptosis in mouse embryos at the 1-4 cell stage (Well et al., 1996) and bovine embryos at the 1-16 cell stage (Matwee et al., 2000). Also mRNA for caspases-2, -3, -6 and -12 are present throughout preimplantation embryonic development in the mouse as are transcripts for the antiapoptotic Bcl-2, Bcl-x_l and Bcl-w and the proapoptotic Bax (Exley et al., 1999). Certain aspects of cell death are altered in early embryos since the time required for staurosporine to induce apoptosis in mouse embryos at the 1-4 cell stage was longer than for blastocysts or other cell types (Well et al., 1996). One possibility is that early embryos have high amounts of antiapoptotic proteins such as Bcl-2 and that heat-induced apoptosis becomes possible when amounts of these proteins decline during development. Indeed, the abundance of mRNA for Bcl-x and Bcl-2 was highest in mouse embryos at the 1-cell stage and decreased as the embryo advanced in development (Juriscova et al., 1998). In contrast, transcripts for Bax and Bad were low in zygotes and two-cell embryos and slowly increased through the blastocyst stage (Juriscova et al., 1998). Moreover, the lack of apoptosis in early embryos at the 1 or 2-cell stage suggest that a minimal number of blastomeres may be required to form a viable embryo.

In other cell types, heat shock activates sphingomyelinases to generate ceramide through the hydrolysis of sphingomyelin (Mirkes and Little, 2000). On the other hand, growth factors stimulate ceramidases and sphingosine kinases to generate sphingosine-1-phosphate which activates the extracellular signal-regulated kinase (ERK) cascade and leads to cell proliferation (Pena et al., 1997). It is possible that the lack of heat-induced apoptosis in early embryos is a result of low ceramide generation due to reduced activation of sphingomyelinases or high concentrations of ceramidases and sphingosine-1-phosphate which might act to block apoptosis.

Even though the capacity for apoptosis is important for the continued development of embryos exposed to stress, it represents an embryo that has been damaged and activated a cell suicide program to remove the affected cells. Therefore, practical manipulation of embryonic apoptotic status as a way to improve embryonic survival is very limited. For example, screen embryos for whether they have undergone apoptosis before transfer would result in lower survival because apoptosis is a sign that embryos might have been stressed. For a similar reason one would not want to activate apoptosis to make embryos in vivo more resistant to heat stress. Thus, activation of apoptosis would only be beneficial if limited to cells that have been damaged.

The second determinant of embryonic resistance to heat shock identified in this thesis is genotype. It is well known that *B. indicus* and certain *B. taurus* breeds such as Senepol are more resistant to elevated temperature and humidity than breeds that evolved in Europe such as Angus and Holstein. Even though a large part of this genetic variation results from the ability of heat-tolerant breeds to regulate body temperature under heat stress (Adeyemo et al., 1979; Bennett et al., 1985; Hammond et al., 1996; Hammond et al., 1998; Gaughan et al., 1999) there is also evidence that heat-tolerant breeds have mechanisms other than body temperature regulation that allow for temperature resistance (Malayer and Hansen, 1990; Kamwanja et al., 1994). A major focus of this dissertation (Chapter 5) was to determine whether embryos from heat-tolerant breeds (Brahman and Senepol) were more resistant to elevated temperature than embryos from heat-sensitive breeds (Holstein and Angus). While poor development precluded conclusions about Senepol embryos, Brahman embryos were better able to survive after exposure to elevated temperature than embryos from Angus and Holstein. This suggests that genetic adaptations exists at the cellular level and that heat-tolerant breeds of cattle have evolved to acquire intrinsic mechanisms for cellular protection against the deleterious effects of high temperature.

The mechanisms responsible for breed differences in cellular resistance to heat shock are not known. Differences in amount of HSPs, which plays a major role stabilizing and refolding damaged proteins (Hartl and Martin, 1992; Becker and Craig, 1994), could account for such differences. However, intracellular amounts of HSP70 were increased in lymphocytes (Kamwanja et al., 1994) and endometrial tissue (Malayer and Hansen, 1990) following heat shock regardless of breed. Moreover, results from chapter 5 demonstrated that heat shock reduced lymphocyte intracellular glutathione content regardless of breed. The lack of breed differences in heat-induced changes in intracellular HSP70 or glutathione content suggest that these molecules are not playing a major role as determinants of cellular thermotolerance among breeds. It is likely that the Brahman has one or more genetic polymorphisms in genes controlling cellular thermotolerance that allow greater functional activity under elevated temperature. It is possible that a secretory heat-induced chaperone protein such as clusterin plays a role in this breed differences in thermotolerance.

Interestingly, Brahman and Senepol lymphocytes were more resistant to heat-induced apoptosis than lymphocytes from Angus and Holstein. Such a result suggests genetic alterations in some aspect of the apoptotic pathway. However, before the significance of breed differences in apoptosis can be understood, it needs to be established whether cells from Brahman and Senepol are more resistant to heat shock because they do not die through apoptosis or if they undergo less apoptosis because cellular physiology is less compromised by heat shock

Identification of genes responsible for heat-tolerance in Brahman cattle may allow these genes to be transferred to heat-sensitive breeds of cattle to result in animals whose oocytes and embryos are more resistant to elevated temperature. Another potential approach to increase embryonic resistance to heat stress would be to inseminate cows from heat-sensitive breeds with semen from heat-tolerant sires. Two experiments argue against this approach, however. First, there were no differences in thermal resistance between Brahman and Holstein embryos at the 2-cell stage (Krininger III et al., 2001). Thus, embryonic gene activation may be required before the embryo can display its genetic potential for thermotolerance and genes conferring thermotolerance would not be activated during the first few days of pregnancy when the embryo is most susceptible to heat stress (Ealy et al., 1993). Secondly, breed of oocyte is a more important determinant of breed differences in heat shock than breed of sire (Block et al., 2002). When oocytes were fertilized with Angus semen, heat shock reduced development to the blastocyst stage in embryos derived from Hosltein oocytes but not in embryos derived from Brahman oocytes. Conversely, when Holstein oocytes were fertilized either with Angus or Brahman semen, heat shock reduced development to the blastocyst stage regardless of breed of sire (Block et al., 2002). These data were interpreted to mean that either thermotolerance genes are paternally imprinted or that some cytoplasmic factor from the oocyte is responsible for genetic differences in embryonic thermotolerance. In either case, the experiment of Block et al. (2002) suggests use of artificial insemination with semen from a heat-tolerant breed probably would not increase resistance of embryos to elevated temperature.

The last series of studies were performed to test whether manipulation of antioxidant status would improve reproductive function and milk yield of lactating dairy cows and in vitro development of bovine embryos after heat shock. The rationale for these experiments was based on the idea that the hyperthermia experienced by heat-stressed cows would cause an increase in metabolism leading to increased oxygen consumption and free radical production. There is evidence that heat stress can deplete plasma antioxidant activity in dairy cattle (Harmon et al., 1997). In addition, vitamin E or GSH could partially alleviate the deleterious effects of heat shock as demonstrated by an increase in viability of mouse morula exposed to 43°C (Aréchiga et al., 1994).

Despite the evidence just cited, efforts to improve fertility in lactating dairy cows using multiple injections of vitamin E and selenium were ineffective regardless of whether cows were bred during hot or cool months (Chapter 6). The possible causes for the failure of vitamin E and selenium to alter fertility has already been outlined in Chapter 6. One question that remains, though, is whether it is warranted to make continued efforts to improve fertility of heat-stressed cows using antioxidant treatments. There are good reasons to be discouraged that such an approach will be effective. The studies testing effectiveness of vitamin E and selenium outlined in Chapter 6 are the latest in a series of generally-disappointing studies on antioxidant treatments. Other antioxidant regimens that were without effect on fertility of heat-stressed dairy cows include injection of 3000 IU vitamin E at the time of artificial insemination (Ealy et al., 1994) and multiple injections of β-carotene on days -6, -3 before artificial insemination and at the time of artificial insemination (Aréchiga et al., 1998b). Feeding supplemental β-carotene for a period ≥ 90 days before artificial insemination had only a slight beneficial effect on reproductive function reflected by an increase in pregnancy rates at 120 days (Aréchiga et al., 1998a).

It is likely that the failure of vitamin E and selenium to improve reproductive function during heat stress reflects the fact that antioxidant status is less important for determining reproductive function in the summer than we had hypothesized. Several studies indicate that oxygen consumption of lactating cows decreases during heat stress (Kibler et al., 1949; Kibler and Brody, 1956; Sorensen and Weniger, 1987) probably because feed consumption and milk yield is reduced, and such an effect would decrease free radical production. Furthermore, experiments demonstrating that addition of vitamin E to culture medium of 2-cell bovine embryos did not protect embryos from heat shock (Chapter 6) are evidence that either lipid peroxidation is not a major mechanism of damage in heat-shocked embryos or that the concentration of vitamin E tested was not able to block oxidative process induced by heat.

It is, of course, possible that failure to improve fertility in heat-stressed cows using antioxidants reflects the fact that the wrong antioxidant was used - that the antioxidants tested so far either target the wrong cellular compartment or are not delivered to the reproductive tract in sufficiently high concentration to block heat-induced free radical production. For example, free radical production during heat shock might be more pronounced in cytoplasmic compartments of the embryo than in membranes. In such a case, vitamin E status would be of less importance than the status of water-soluble antioxidants such as GSH. Stimulation of GSH synthesis by adding S-adenosyl-methionine to culture medium of mouse morulae has been shown to decrease the deleterious effects of 43°C on embryonic viability and development to the blastocyst stage (Aréchiga et al., 1995) while direct addition of GSH to the culture medium of heat-shocked embryos increased embryo viability (Aréchiga et al., 1994). There are also antioxidants such as α-lipoic acid which is able to act in either water- or lipid-soluble compartments of the cell (Packer, 1998) and may therefore be more efficient to prevent cellular damage caused by heat shock.

Taken together, the work outlined in this thesis indicates that not all embryos are equal with respect to resistance to heat stress. Acquisition of resistance to heat shock is controlled by genotype and developmental processes including the ability of embryos to undergo apoptosis. It may prove possible to manipulate cellular determinants of thermotolerance to improve embryonic resistance to stress and increase fertility in lactating dairy cows

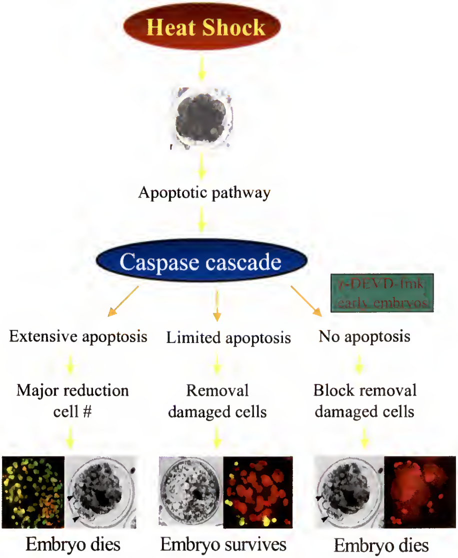

Figure 7-1. Heat-induced apoptosis in bovine embryos. The fate of the embryo depends on the degree of apoptosis. Exposure of bovine embryos to a stress such as heat shock activates the apoptotic pathway and caspase cascade leading to cell death by apoptosis. Exposure to a severe heat shock may cause massive apoptosis and major reduction in embryo cell number to result in embryo death. In the other extreme, the inability to undergo apoptosis in response to heat shock observed in early embryos at the 2-8 cell stage or in embryos pharmacologically treated with a caspase inhibitor z-DEVE-fmk may prevent the removal f heat-damaged cells and the embryo will die too. In contrast, the embryonic ability to respond to heat shock by undergoing limited apoptosis may allow the removal of heat-damaged blastomeres and continued development.

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BIOGRAPHICAL SKETCH

Fabíola Freitas de Paula Lopes was born on January 29, 1970, in Recife, State of Pernambuco, Brazil. She was the youngest child of Maria Odete and João Paula Lopes. As a child, she spent her vacations on her uncle's farm and helped him with the animals. In 1987, Dr. Paula Lopes graduated from high school in Recife. In 1988, she entered the School of Veterinary Medicine at the Universidade Federal Rural de Pernambuco (UFRPE). While attending the School of Veterinary Medicine, Dr. Paula Lopes worked as a trainee at the Bovine Clinics in Garanhuns, an experimental station that provides veterinary services for the dairies in the State of Pernambuco. Dr. Paula Lopes graduated in 1992 and began her residency program, where she was exposed to an intensive clinical practice and to reproduction of large animals. After completion of her residency program, Dr. Paula Lopes participated in a research project to study the prevalence of tuberculosis in the state of Pernambuco, under the supervision of Dr. José Augusto Bastos. In 1994, Dr. Paula Lopes was married to Mauricio Domingues Coutinho Neto. In the same year, she moved to the USA where her husband began a PhD at the University of Pittsburgh. In 1995, she moved to Florida and began her graduate studies in the area of reproductive biology at the Department of Dairy and Poultry Sciences under the supervision of Dr. Peter J. Hansen. In 1998, she began a PhD program in Animal Molecular and Cell Biology under the supervision of Dr. Peter J. Hansen. Upon completion of the requirements of the Doctor of Philosophy degree, she will move to Germany and pursue a postdoctoral position at the Institute of Molecular Animal Breeding, Ludwig Maximilian Universität, Munich.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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